

**SELECT NUTRIENTS, SECRETED PHOSPHOPROTEIN 1 AND INSULIN-
LIKE GROWTH FACTOR 2: EFFECTS ON TROPHECTODERM OF OVINE
CONCEPTUSES**

A Dissertation

by

JIN YOUNG KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Physiology of Reproduction

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ABSTRACT

Select Nutrients, Secreted Phosphoprotein 1 and Insulin-Like Growth Factor 2: Effects
on Trophectoderm of Ovine Conceptuses. (May 2010)

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Histotroph, secretions from luminal (LE), superficial glandular (sGE) and glandular (GE) epithelia and molecules selectively transported into the uterine lumen, are essential for peri-implantation ovine conceptus development and maternal recognition of pregnancy. Among them, several components of histotroph including nutrients, cell matrix proteins and growth factors may activate mTOR (mammalian target of rapamycin; also known as FRAP1) to stimulate hypertrophy, hyperplasia, and/or migration of conceptus trophoctoderm cells, as well as expression of IFNT for pregnancy recognition and critical proteins for conceptus development. Therefore, studies were conducted to examine effects of select nutrients (arginine, leucine, glutamine and glucose), IGF2 and SPP1 on mTOR signal transduction pathways and determine their biological effects on proliferation, migration and/or attachment of ovine trophoctoderm (oTr) cells and conceptuses (embryo and its extra-embryonic membranes).

The first study defined the expression of *IGF2*, *RPS6K*, phosphorylated AKT, RPS6K, P38 and ERK1/2 MAPK by the uterus and conceptus during the peri-implantation period. In addition, effects of IGF2 on the PI3K signaling pathway were evaluated using oTr cells isolated from Day 15 conceptuses. *IGF2* was most abundant in compact stroma of endometrial caruncles and also present in all cells of the conceptus, but particularly abundant in the endoderm and yolk sac. Phosphorylated AKT1, RPS6K, P38 and ERK1/2 proteins were abundant in nuclei of endometrial LE and conceptus

trophectoderm. IGF2 activated multiple cell signaling pathways including PDK/AKT/mTOR/RPS6K and MAPKs that are critical to survival, growth and migration of the ovine trophoblast cells.

The second study demonstrated the multifunctional effects of secreted phosphoprotein 1 (SPP1) on oTr cells including cell signaling transduction, migration, and adhesion. Novel results of this study indicated that SPP1 binds $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrins to activate PI3K/mTOR/RPS6K, MAPK as well as crosstalk between mTOR and MAPK pathways that are essential for expansion and elongation of conceptuses and attachment of trophoctoderm to uterine LE during implantation.

The third study identified effects of arginine (Arg), leucine (Leu), glutamine (Gln) and glucose on oTr cells. Arg, Leu and glucose, but not Gln, activated PI3K-AKT1 and mTOR-RPS6K-RPS6 signaling pathways. Arg, Leu and glucose increased abundance of p-RPS6K in nuclei and p-RPS6 in cytoplasm of oTr cells. In addition, results of this study demonstrated that Arg and Leu are remarkably stimulatory to cell proliferation and migration.

The fourth study determined effects of Arg on signal transduction pathways and oTr cell proliferation, as well as inhibition of oTr cell proliferation by L-NAME (an inhibitor of NOS) or Nor-NOHA (an inhibitor of arginase) on oTr cells. Arg increased p-mTOR, RPS6K and 4EBP1 protein and also increased protein synthesis and reduced protein degradation in oTr cells. Both NO and polyamines enhanced cell proliferation in a dose-dependent manner. The effects of Arg were partially inhibited by both L-NAME and Nor-NOHA. These results indicate that Arg enhances production of polyamines and NO and activates the mTOR-FRAP1-RPS6K-RPS6 signaling pathway to stimulate proliferation of oTr.

The fifth study identified differential effects of Arg, Leu, Gln and glucose on gene expression and protein translation in explants cultures of ovine conceptuses. Expression of mRNAs was not affected by treatments with the select nutrients; however, Arg, Leu, Gln and glucose increased abundance of total and phosphorylated forms of

mTOR, RPS6K, 4E-BP1 and RPS6. Arg, Leu, Gln and glucose also increased the amounts of NOS and ODC1, but only Arg stimulated a significant increase in abundance of IFNT.

Collectively, these studies indicated that IGF2, SPP1 and select nutrients activate mTOR cell-signaling pathways that converge on AKT1 and that are likely critical to mechanism(s) responsible for survival, elongation and development of conceptuses. A more complete understanding of this mechanism will be important to development of strategies to reduce early embryonic losses in ruminants and in other species including humans.

DEDICATION

To my family

ACKNOWLEDGEMENTS

I would like to thank my mentors, Drs. Fuller W. Bazer and Thomas E. Spencer, for their support, patience, encouragement and advice throughout the course of this research. They have taken time out of their busy schedules to guide me in my research efforts, help me troubleshoot problems, and suggest where to focus my research interests. I would also like to thank my committee members, Drs. Robert Burghardt, Gregory Johnson, and Guoyao Wu. I greatly appreciate the time and effort they put into helping me to become a well rounded scientist. Thanks also to the members of the Laboratory for Uterine Biology and Pregnancy for their assistance and friendship. I would like to express my gratitude to my family and friends.

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CHAPTER I

INTRODUCTION

Establishment of pregnancy requires complex interactions and communication between the maternal endometrium and the developing conceptus (embryo/fetus and associated extraembryonic membranes). During the peri-implantation period, development of conceptus is supported by uterine secretions, referred to as histotroph, which are primarily regulated by progesterone (1-3). Histotroph is a complex mixture of a number of factors including ions, amino acids, sugars, growth factors, enzymes, adhesion proteins, cytokines, hormones, and transport proteins that are required for growth and development, and implantation of the conceptus (4). Results from the uterine gland knockout ewe (UGKO) established that secretions of the endometrial GE are the main source of histotroph and essential for peri-implantation conceptus elongation and survival (5, 6).

FRAP1 (FK506 binding protein 12-rapamycin associated protein 1, also formerly known as mTOR) is a key component of a signaling pathway that regulates cell growth and development in response to amino acid levels, energy sufficiency and growth factors (7-9). FRAP is a giant serine-threonine protein kinase, which forms a complex with scaffold protein raptor (MTORC1) or rictor (mTORC2) to regulate 70-kDa ribosomal protein S6 kinase 1 (RPS6K, also known as p70S6K1), and the eukaryotic translation initiation factor 4E binding protein 1 (eIF4E-BP1) as well as cytoskeleton reorganization. The best characterized cell signaling molecules downstream of MTORC1 are RPS6K and eIF4EBP1. Phosphorylation of RPS6K enhances the translation of mRNA by activation of ribosomal proteins and translation elongation factors. 4E-BP1, which binds to eIF4E and represses translation initiation, is relieved of repressive effects following phosphorylation by mTOR. The mTORC2 has an independent, rapamycin-resistant function which is implicated in the control of actin cytoskeleton dynamics through activated AKT or Rho cell signaling (10).

This dissertation follows the style of Endocrinology.

Disruption of the *FRAP1* gene leads to early post-implantation lethality due to impaired cell proliferation and hypertrophy in both the inner cell mass and trophoblast (11, 12). In addition, ablation of *Raptor*, *mLST8* (13), *Rictor* (13, 14), and *Mapkap1* (15) genes results in disfunction of mTORC1 and mTORC2, respectively with fetal lethality occurring at different stages of development. These studies suggest that both FRAP1 complexes are required for embryonic and trophoblast development.

Components in uterine secretions, including amino acids and glucose, may regulate FRAP1 activity in conceptus development. In mouse embryos, leucine (Leu), arginine (Arg) and glutamine (Gln) uptake is increased (16) at the time of implantation and other hormones may regulate trophoblast differentiation through a classical FRAP1-dependent pathway (17, 18). In the absence of amino acids, growth factor signals have little or no impact on mTORC1 signaling (17) indicating the significance of amino acids as regulators of FRAP1. Glucose itself and glucose metabolites may regulate the activity of FRAP1 via insulin and insulin-initiated signaling (19, 20) or via an insulin-independent pathway to regulate proliferation of trophoblast cells (21). Further, secreted phosphoprotein 1 (SPP1) is the secreted ECM protein in histotroph that may activate the mTORC2 complex through signaling that converges with nutrient-activated signaling pathways to increase trophoblast cell migration.

Collectively, available results established that the FRAP1 is important in conceptus development through effects on translation of mRNAs for proteins required for growth, proliferation and differentiation of the trophoblast (22) as well as mobility of cells for adhesion and attachment which is required for synepitheliochorial placentation. However, the mechanism whereby FRAP1 activity is regulated in ovine conceptuses and uteri remains poorly understood. Therefore, the present studies were conducted to identify molecular mechanisms and biological functions of FRAP1 with respect to responses of ovine trophoblast to nutrients (amino acids and glucose), a growth factor (IGF2) and an extra cellular matrix protein (SPP1), each of which may be critical for conceptus growth and development in sheep.

CHAPTER II

LITERATURE REVIEW

Conceptus Development in Sheep

In sheep and other ruminants, implantation is classified as synepitheliochorial due to variations in the length of the different phases of implantation and the degree of endometrial invasion by the trophoblast (23-25). Developmental events of the pre-implantation conceptus are achieved in four steps including cleavage, compaction, shedding of the zona pellucida and rapid elongation. In response to a surge of Luteinizing Hormone from the anterior pituitary, ovulation of the oocyte occurs approximately 24-30 h after onset of estrus (26). The unfertilized ovum enters the oviduct and is fertilized by sperm at the ampullary-isthmic junction. Following fertilization, the zygote (27) surrounded by a mucopolysaccharide coat, the zona pellucida, travels through the isthmus of the oviduct and undergoes multiple mitotic divisions (28-30). During this period, the first cleavage to the 2-cell stage occurs approximately 39 to 52 h post-breeding, the second cleavage to the 4-cell stage at 52 to 75 h and the third cleavage to the 8-cell stage at 75 h or 3 days post-breeding (29). Between Days 4 and 5, the embryo undergoes compaction and it becomes difficult to count individual blastomeres (termed the morula stage) and it enters the uterine lumen (Fig. 2.1). There are two distinct cell populations known as inner cell mass which gives rise to the fetus and trophoblast that gives rise to the placenta. When the morula is formed, inner cells of the morula develop gap junctions between adjacent cells that increase intercellular communication and coordination. The outer cells of morula begin to be compacted by forming cell-cell adhesions known as tight junction that alter their polarity and permeability, resulting in accumulation of a fluid in a central cavity called the blastocoele (27-32). The blastocoele is surrounded by trophoblast cells while the inner cell mass is localized to a single pole of the blastocyst. At this time, the embryo is called a blastocyst that contains approximately 3000 blastomeres and is 150 to 200 μm in diameter (28, 32). As the blastocyst continues to grow by mitotic divisions, fluid fills the blastocoele and increases the volume and pressure, resulting in stretching of the zona

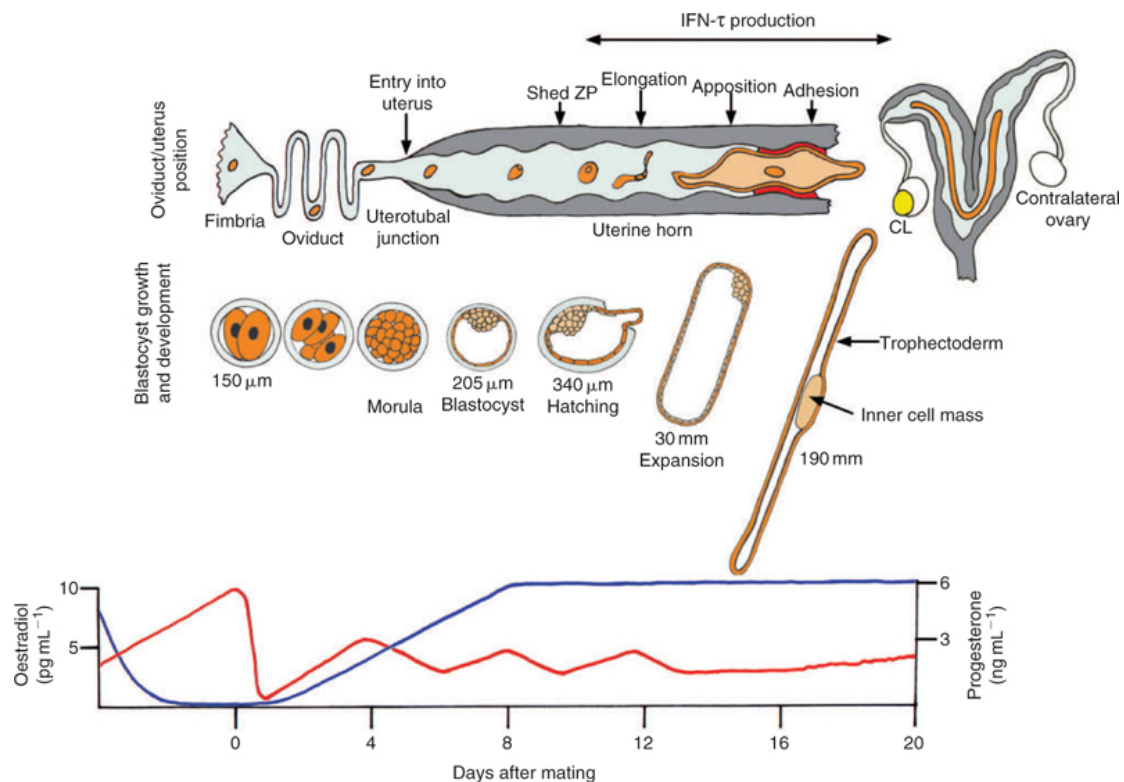


Fig. 2.1. Early pregnancy events in sheep. The embryo enters the uterus on Day 4 after mating (Day 0 = estrus/mating) at the morula stage of development and then develops into a blastocyst on Day 6. Between Days 8 and 9, the blastocyst emerges from the zona pellucida as lysis occurs in response to enzymes such as uterine and/or embryonic proteases. After Day 10, the blastocyst elongates into a tubular and then into a filamentous conceptus, and then appears to be immobilized in the uterine lumen becoming closely associated with the endometrial luminal epithelium (LE) followed by unstable adhesion. Between Days 16 and 22, the trophoblast begins to adhere firmly to the LE by interdigitation between uterine epithelial microvilli and projections of the trophoblast cells, and/or penetration into the superficial duct of the uterine glands (sGE) by papillae of the trophoblast. During this time, the trophoblast giant cells migrate, appose, and fuse to the apical surface of the endometrial LE to form syncytial plaques. Eventually, as a part of synepitheliochorial placentation in sheep, the syncytial plaques cover the caruncular surface and aid in formation of the placentome which are structures formed by fusion of placental cotyledons and endometrial caruncles. Adapted from Spencer et al., 2007 and originally drawn by Dr. Greg A. Johnson.

pellucida occurs in response to proteolytic enzymes from the trophectoderm, leaving the blastocyst surrounded by monolayer of trophoblast cells and establishment of an internal layer of endodermal cells, the extra-embryonic endoderm, separated by a basement membrane (28, 30-33).

Once the zona pellucida is shed, the blastocyst continues to expand and undergo a morphological transition from a spherical to tubular conceptus (embryo and its associated extra-embryonic membranes) on Day 12 measuring 11 mm and then becomes an elongated filamentous conceptus of about 100 mm by Day 14 and 150 to 190 mm on Day 15 while the width remains approximately 1 to 1.5 mm throughout the period of elongation (23, 28-30, 32, 33). In sheep, embryonic development to the morula and blastocyst stage is not entirely dependent on the uterus (34, 35). However, blastocysts or trophoblastic vesicles, derived from Day 12 conceptuses survive, but are unable to undergo elongation *in vitro*. When these trophoblastic vesicles are transferred into the uterus the trophoblast elongates and produces interferon tau (IFNT), the pregnancy recognition signal in ruminants (36-39). This evidence indicates that ovine blastocyst elongation is a uterine dependent event and may be species-specific, because in rodents, trophectoderm growth requires the presence of the inner-cell mass (37) (see Fig. 2.1).

IFNT During the Peri-Implantation Period in Sheep

In ruminants, elongation of the blastocyst results in production of IFNT which is the pregnancy recognition hormone (38-40) that acts in paracrine manner on the uterine endometrial epithelia to prevent development of the luteolytic mechanism, thereby maintaining a functional ovarian corpus luteum (CL) for continued production of progesterone (P4) (41-44). This antiluteolytic effect of IFNT is required for successful establishment of pregnancy and development of the conceptus.

During the peri-implantation period, IFNT is synthesized and secreted by the mononuclear trophectodermal cells of conceptus between Days 10 and 21-25 (maximally on Days 13-16) (39, 40, 45, 46). Maximal secretion of IFNT by the trophoblast is directly related to the stage of development of the blastocyst or morphological transition of the conceptus from spherical to filamentous forms (45, 47).

As the pregnancy recognition signal molecule, IFNT acts directly on the endometrial LE and sGE to suppress transcription of *ESR1* which then prevents estrogen-induced expression of the oxytocin receptor (*OXTR*) gene (44, 48), thereby inhibiting oxytocin-induced pulsatile release of prostaglandin F2 α (PGF) from the uterine epithelium (Fig. 2.2). During the normal estrous cycle, expression of the progesterone receptor (*PGR*) decreases in LE and shallow GE while expression of estrogen receptor alpha (*ESR1*) increases after about Day 13 and then estradiol (E2) induces *OXTR* expression from Days 14 to 16 (49, 50), thereby allowing oxytocin from the posterior pituitary and/or CL to induce release of luteolytic pulses of PGF on Days 15 to 16 (51).

In contrast, during early pregnancy, IFNT from elongating conceptus suppresses *ESR1* expression which prevents E2-induced *OXTR* expression (52-56). Collectively, these antiluteolytic actions of IFNT prevents increases in epithelial *ESR1*, *PGR*, and *OXTR* gene expression, which are all E2 responsive, by directly inhibiting transcription of the *ESR1* gene, thus maintaining secretion of P4 by the CL (48, 57, 58). In addition to its anti-luteolytic effects, IFNT also induces or enhances the expression of a number of genes within the endometrium that are hypothesized to support establishment of apical interactions between trophoblast and epithelial cells during early pregnancy (41, 58-60). A subpopulation of mononuclear trophoblast cells become binucleated between Days 14 and 16 and begin to migrate and differentiate to form syncytia with uterine LE, resulting in loss of endometrial LE (25). Giant binucleate trophoblast and multinucleated syncytiotrophoblast cells also alter endometrial gene expression by secretion of placental hormones such as placental lactogen (CSH1) and pregnancy associated glycoproteins (PAG) (25, 61-63) (see Fig. 2.2).

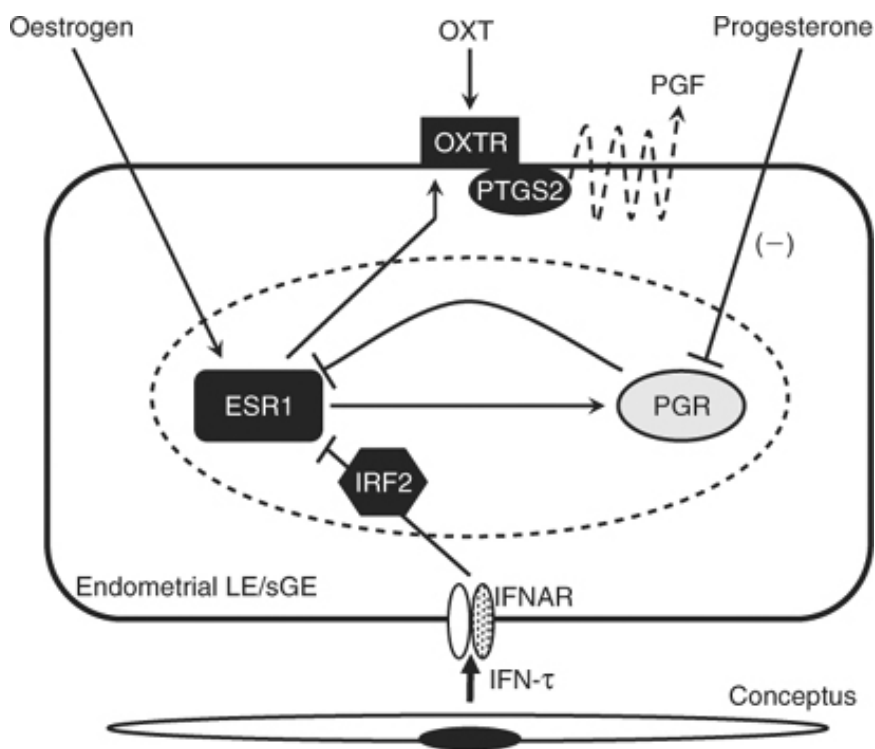


Fig. 2.2. Schematic illustrating current working hypothesis on IFNT signaling in the ovine endometrial stroma and glandular epithelium. During the peri-implantation period, ovine IFNT is synthesized and secreted by the mononuclear trophectodermal cells between Days 10 and 21-25 (maximally on Days 14-16) and acts directly on endometrial LE and sGE to suppress transcription of *ESR1* and *OXTR* genes, thereby preventing production of luteolytic pulses of PGF. During the estrous cycle, *ESR1* expression increases and *PGR* expression decreases on Days 11 to 13 and then E2 induces *OXTR* expression on Days 13 to 14, thereby allowing oxytocin from the posterior pituitary and/or CL to induce release of luteolytic pulses of PGF on Days 15 to 16. In contrast, during early pregnancy, secreted IFNT from fully elongated conceptus silences *ESR1* expression which prevents E2-induced *OXTR* expression. However, IFNT does not stabilize *PGR* expression in endometrial epithelia during pregnancy. Adapted from Spencer et al., 2007.

The Stage of Implantation of Conceptuses

Ruminant (sheep, cattle, and goats) and pig conceptuses do not invade into the maternal endometrium, but superficially attach to the endometrium LE. After attachment, placentomes form by interdigitation between maternal caruncles and placental cotyledons, which allow transport of hematotrophic nutrition via placentomes in ruminants. The stages of implantation in ruminant species include: 1), shedding of the zona pellucid; 2) precontact and blastocyst orientation; 3) apposition; 4) adhesion; and 5) endometrial fusion which differs markedly from implantation schemes in species with invasive implantation such as primates and rodents (Fig 2.3) (23, 24, 64). During the period of conceptus elongation, apposition of the conceptus trophectoderm and endometrial LE is initiated on Day 14 followed by attachment and interdigitation of trophectoderm cells and microvilli of the LE (24, 64). During attachment, this interdigitation ensures firm adhesion of the fetal maternal interface in both caruncular and intercaruncular areas on Day 16 which makes it impossible to recover the conceptus intact from the uterine lumen via surgical flushing by Day 18 of pregnancy. During this stage in sheep, trophoblast giant binucleate cells begin to fuse apically with the endometrial LE to form syncytia, thereby assimilating and replacing the endometrial LE (65) (see Fig. 2.3).

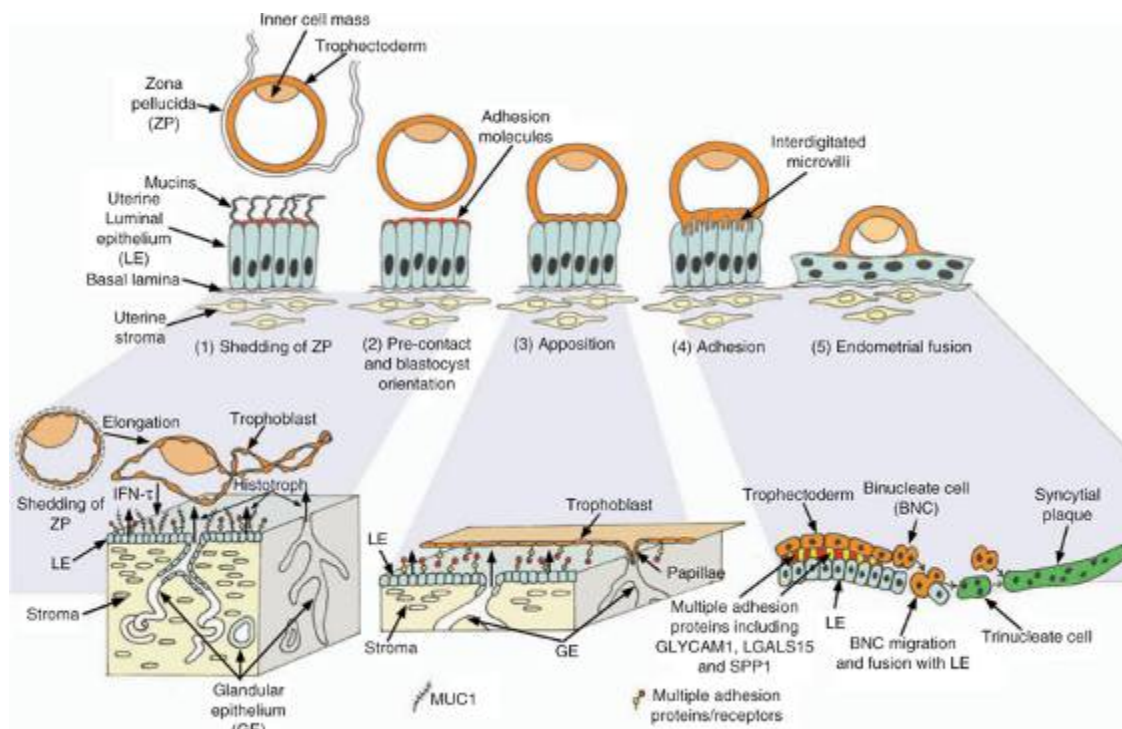


Fig. 2.3. The phases of blastocyst implantation in sheep. Shedding of the zona pellucida (Phase 1): The embryo enters the uterus on Day 4. The blastocyst is formed on Day 6 and the zona pellucida is shed on Day 8 or 9 due to blastocyst growth and uterine and/or embryonic proteases. After Day 10, the blastocyst elongates and develops into a tubular and then into a filamentous conceptus. Precontact and blastocyst orientation (Phase 2): Between Days 9 and 14, there is no definitive cellular contact between the conceptus trophoblast and the endometrial epithelium, but the blastocyst appears to be positioned and immobilized in the uterus. During this time, elongation of the blastocyst plays an important role in developmentally regulated production of IFNT in sheep. Apposition (Phase 3): The conceptus trophoblast associates closely with the endometrial LE followed by unstable adhesion. In ruminants, the trophoblast develops finger-like villi or papillae and thereby penetrates into the superficial ducts of the uterine glands. This event has been hypothesized to anchor the peri-attachment conceptus and allow it to absorb histotroph from uterine glands. Adhesion (Phase 4): On Day 16, the trophoblast begins to adhere firmly to the endometrial LE. The interdigitation of the trophoblast and endometrial LE occurs in both the caruncular and intercaruncular areas of the endometrium. During this time, the mononuclear trophoblast cells differentiate into trophoblast giant binucleate cells. Adapted from Spencer et al., 2007 and originally drawn by Dr. Greg A. Johnson.

The Requirement for Histotroph for Conceptus Development in Sheep

During early pregnancy, interactions and communication between conceptus and various endometrial cells, especially LE, GE, and stroma, are essential to coordinate conceptus development, uterine angiogenesis, maternal recognition of pregnancy, and transport of amino acids and glucose into the uterine lumen (1, 63, 66). To support these key events of pregnancy, mammalian uterine glands produce and secrete complex array of molecules referred to as histotroph. Histotroph include growth factors, ions, amino acids, mitogens, nutrient transport proteins, hormones, enzymes, proteases, protease inhibitors, glucose, cytokines, lymphokines, and many other substances (1, 3, 67-69). The function of these secretions may differ depending upon the type of epithelium from which they originate. Secretions from the LE appear to assist in implantation, while those from GE may aid in development and nutrition of post-implantation conceptuses (3, 69, 70). The importance of these secretions has been demonstrated to be essential in both primate and subprimate species as regulators of conceptus development and survival, pregnancy recognition, and implantation/placentation (5, 6, 32, 71, 72).

Compared to events in domestic livestock, the period of pre-implantation embryonic development is extremely short in rodents. Although the pre-implantation period is short, evidence from studies of mice in which genes have been knocked out identified several factors in histotroph, including leukemia inhibitory factor (73) and calcitonin, that are only secreted by the uterine glands and are required for successful establishment of uterine receptivity and implantation of blastocysts (74, 75). The prolonged nature of pre-implantation conceptus development in domesticated species such as the pig, cow, horse, and sheep suggests that endometrial support via histotroph is particularly critical in these species.

Studies of the uterine gland knockout ewe (UGKO) revealed an essential role for secretions from uterine glands for regulation of luteolysis during normal estrous cycles, early conceptus development and initiation of pregnancy recognition signaling (5, 6, 76). The UGKO ewe model is produced by the administration of a synthetic, non-metabolizable progestin to neonatal ewe lambs during the critical period of endometrial gland morphogenesis from birth to postnatal Day 56 (77). This early exposure to a progestin permanently ablates development of GE without apparent defects in development of the myometrium or other Müllerian duct-derived structures in the female

reproductive tract or function of the hypothalamic-pituitary-ovarian axis (77, 78). The adult UGKO ewes are unable to experience normal estrous cycles or to support early embryonic development for blastocyst elongation due to the absence of endometrial glands and a markedly reduced uterine LE surface area. Transfer of hatched blastocysts from normal synchronous ewes into the uteri of UGKO recipient ewes also failed to ameliorate the pregnancy defect (6). Further, blastocysts can be found in the uterine flushings of bred UGKO ewes on Day 9 of pregnancy suggesting that early conceptus development does not require uterine secretions (5, 6). In UGKO ewes, blastocysts hatch from the zona pellucida around Day 8 as expected; however, there is failure of blastocyst elongation and ultimately the conceptuses die or they are severely growth-retarded by Day 14 of pregnancy.

In UGKO ewes, molecular components in uteri that were not altered included steroid hormone receptors, the anti-adhesive factor mucin 1 (MUC1), integrins on the surface of LE, and IFNT responsiveness of the endometrium (5, 76). However, uterine flushings recovered from UGKO ewes on Day 14 contained either very low or undetectable amounts of galectin-15 (LGALS15), glycosylated cell adhesion molecule 1 (GLYCAM1), and secreted phosphoprotein 1 (SPP1, also known as osteopontin), which are adhesion molecules that regulate apposition and attachment of trophectoderm to the LE (5, 79, 80). These results indicated that failure of blastocysts to elongate was due to a lack of uterine glands and their secretions which are essential for peri-implantation conceptus growth and survival. Further, unidentified components of histotroph are absent or reduced in flushings from UGKO ewes and may affect the fertility of these ewes. Similarly, neonatal exposure to a progestin alters uterine morphology and luminal protein content resulting in a reduction or failure in successful establishment and/or maintenance of pregnancy in adult UGKO cows (81).

Indeed, uterine histotroph is a complex fluid which makes it complicated to analyze specific components as to whether they originate from the endometrium or the conceptus. Two-dimensional gel electrophoresis identified a number of proteins in uterine histotroph in a number of species including ewes and cows (45, 82-87); however, establishment of their identity and function has been limited. Recently, quantitative proteomic approaches and mass spectrometry enabled the identification of proteins in

uterine histotroph or components of the endometrial proteome, but these techniques have yielded limited findings despite the improved protein sequence databases. Among them, Berendt et al. (88) reported an increase in four endometrial proteins associated with conceptus–maternal interactions in cattle including Rho GDP dissociation inhibitor beta, 20 alpha-hydroxysteroid dehydrogenase, soluble NADP⁺-dependent isocitrate dehydrogenase 1, and acyl-CoA-binding protein. Uterine histotroph also contains lipids, amino acids and sugars. In pigs, non-protein components of histotroph include amino acids, lipids, and sugars that undergo distinct changes in the uterine lumen as well as fetal fluids as pregnancy progresses (89-91). Glucose and amino acids have important roles for pre-implantation embryonic development (92-95). Both amino acids and their transporters have been implicated in blastocyst implantation and differentiation of the trophoblast through modulation of nutrient-sensing pathways (22, 95-97).

Cellular and Molecular Aspects of mTOR Cell Signaling

mTOR/FRAP1 (FK506-binding protein 12-rapamycin complex-associated protein 1; formerly known as mTOR) is a highly conserved serine/threonine protein kinase that senses and responds to changes in amino acid levels and energy, as well as hormones, mitogens and stress (7, 9, 98, 99), and has a critical role in cell metabolism and growth. FRAP1 and associated proteins comprise two distinct signaling complexes, referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (13, 98, 100) (see Fig. 2.4). The mTORC1 complex consists of RAPTOR (regulatory-associated protein of TOR) and LST8 (also known as G protein β subunit-like) (101-104), while mTORC2 consists of RICTOR (rapamycin-insensitive companion of mTOR), MAPKAP1 (mitogen-activated protein kinase-associated protein 1), and LST8 (105, 106). The FRAP1-associated proteins seem to determine the specificity of the different cell signaling pathways (107-110) mediated by mTOR complexes, associated regulators, and effectors. The mTORC1 controls cell proliferation in part by phosphorylating S6Kinase 1 (S6K1) and the eIF-4E-binding protein 1 (4EBP1), known regulators of protein synthesis. The mTORC2 regulates actin cytoskeleton organization and Akt/PKB phosphorylation (108), which regulates cell migration, growth, survival, and metabolism.

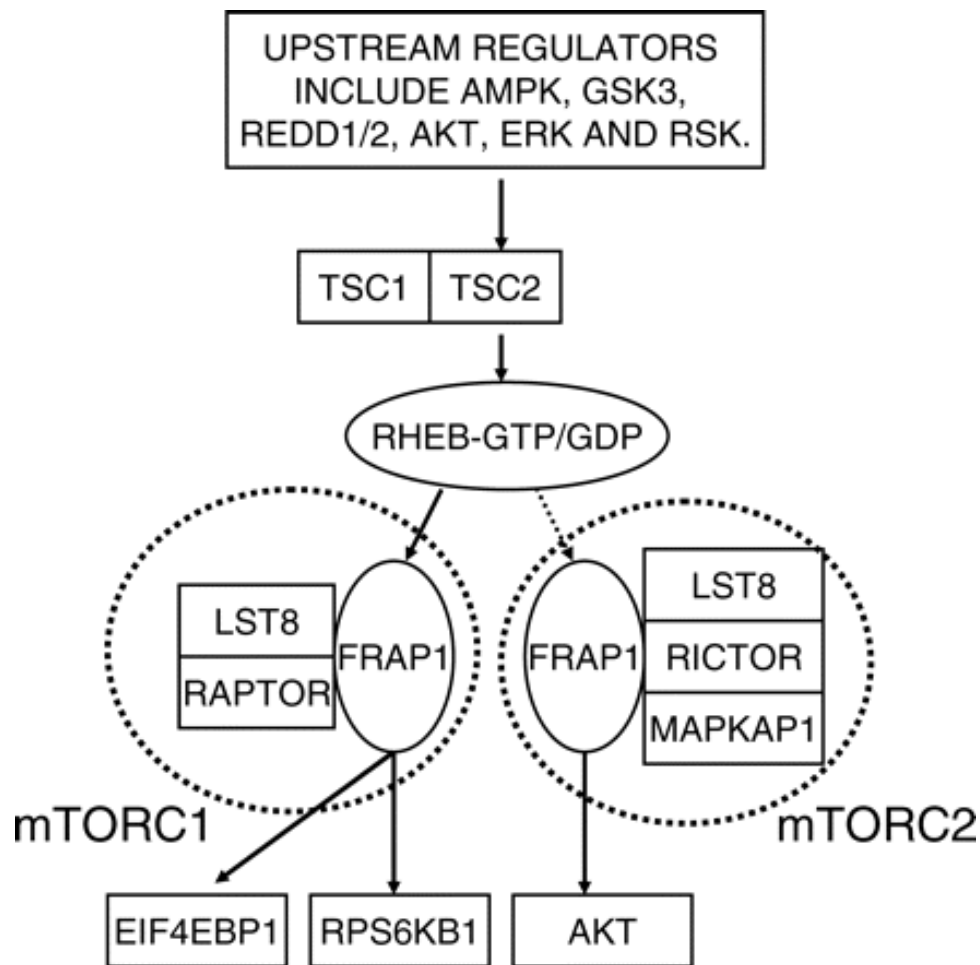


Fig. 2.4. The mTOR complexes include mTORC1 and mTORC2. mTORC1 comprises FRAP1, LST8, and RAPTOR, and its known effectors include EIF4EBP1 and RPS6KB1. mTORC2 comprises FRAP1, LST8, RICTOR, and MAPKAP1, and its known effector is AKT. Under the control of the upstream regulators, the TSC1/TSC2 complex (which has GTPase-activating properties) regulates conversion between GTP and guanosine diphosphate (GDP) and, therefore, the activity of RHEB. RHEB-GTP activates FRAP1 in mTORC1 (and possibly mTORC2). Adapted from Gao et al., 2009 (111).

FRAP1 and associated members of the mTORC1 and mTORC2 cell signaling pathways are likely critical for growth and development of the conceptus, as well as implantation. Disruption upstream of FRAP1, such as TSC, blocks functioning of mTORC1 and mTORC2 and impairs cell proliferation (hyperplasia) and hypertrophy (hypertrophy) in the embryonic disc and in the trophoblast, resulting in post-implantation lethality of blastocysts (14, 15, 112). In mice germ-line disruption of mTOR causes embryonic lethality at early or post-implantation stages of development (11, 12). FRAP1 may also affect conceptus growth by regulating translation of proteins in the uterus, including insulin-like growth factor 2, ornithine decarboxylase 1, and nitric oxide synthases (22, 113, 114). In addition, FRAP1 promotes translation of 5'-terminal oligopyrimidine tract mRNAs (115) that are critical to fetal and placental development (116-118), as well as development, differentiation, and motility of trophoblast cells (22, 96, 119).

During pregnancy, substantial synthesis and secretion of histotroph by uterine glandular epithelium (GE) occurs in response to the conceptus or conceptus-derived pregnancy recognition signals, to provide sufficient nutrients (61, 68). In this system, mTOR cell signaling is the central regulator since available evidence indicates that mTOR regulates the expression and trafficking of glucose transporters (120-122) and amino acid transporters (123, 124). Further, FRAP1 and associated proteins including raptor, rictor and RHEB are localized to uterine LE and GE and trophoblast cells in the ovine conceptus between Days 13 and 18 of pregnancy. Therefore, the mTOR cell signaling complexes likely regulate conceptus development during the peri-implantation period, particularly in species with an epitheliochorial or synepitheliochorial type of placentation characterized by a rapid elongation of conceptus trophoctoderm and a protracted period of implantation (23).

Tumor suppressors (TSC) and RHEB

Most upstream regulators of mTOR appear to function through the tumor suppressor Tuberous Sclerosis Complex 1 (TSC1) and Tuberous Sclerosis Complex 2 (TSC2). Functionally, TSC1 is thought to be the regulatory component, while TSC2 is thought to be the catalytic component (125). In the typical mTOR cell signaling pathway,

TSC1 and TSC2 form a heterodimer to inhibit mTORC1 (109), which functions as a glutamyl transpeptidase (GTPase)-activating protein toward RHEB to stimulate mTORC1 activity (110, 112). Phosphorylation of TSC2 by various upstream protein kinases such as AKT/PRKB and MAPK3 ablates the inhibitory effects of TSC1/TSC2 on mTORC1. In contrast, their functions with respect to mTORC2-mediated cell signaling have not been well investigated, except to demonstrate that mTORC2 phosphorylates AKT/PRKB-FOXO and PRKCA (13). Results of studies in mice devoid of *4932417H02Rik* (*Raptor*), *Gbl* (*Lst8*) (13), *4921505C17Rik* (*Rictor*) (13, 14), *Mapkap1*(15), and *Frap1* indicate that components of mTORC1 and mTORC2 are required for embryonic development, although the timing of formation of these functional complexes may differ (13).

Downstream targets of mTORC1

Downstream of mTOR, two of the most well characterized targets are S6K1 and 4EBP-1 (99). As a result of tight regulation of these two proteins by mTOR, they are often used as functional markers of mTOR activity. S6K1, which is phosphorylated and activated by mTOR, phosphorylates the ribosomal S6 protein which is a component of the 40S ribosomal subunit. Activation of S6 leads to increased ribosomal biogenesis to recruit translational components. Interestingly, mutational loss of the S6K1 phosphorylation sites on S6 leads to increased global protein translation without increasing the percentage of ribosomes engaged in the polysomes (20). On the other hand, 4EBP-1 is inactivated by mTOR phosphorylation. The 4EBP-1 in its hypophosphorylated form binds to and inactivates eIF4E, which is responsible for nuclear cap binding complex (CAP)-dependent translation (9, 126). Therefore, inactivation/phosphorylation of 4EBP-1 by mTOR increases CAP-dependent protein translation.

IGF II

Insulin-like growth factors (IGFs), including IGF-I and IGF-II, are small polypeptide hormones with an approximate molecular weight of 7 kDa (127). They are members of the structurally related insulin/IGF super family. Mature IGF-I and IGF-II

peptides consist of A, B, C, and D domains. The A and B domains of IGFs are homologous to the A and B domains of insulin, and the C domains of IGFs share sequence homology to the C peptide of proinsulin which is cleaved off in mature insulin during prohormone processing. IGFs contain an additional D domain, which is not found in insulin (128, 129).

Both IGF-I and -II act as endocrine, paracrine and autocrine factors in various cell types to stimulate proliferation, survival and differentiation. Their effects are mediated by binding to Type I IGF receptor (IGF-IR), insulin receptors (IRs) and Type II IGF receptors (IGF-IIR), which are tetrameric receptors with two extracellular α -subunits containing the ligand binding domain, and two β -subunits with a tyrosine kinase domain (130). Upon ligand binding, the activated receptors recruit and phosphorylate scaffold proteins, such as insulin receptor substrate 1 (IRS-1) and IRS-2, leading to the activation of several intracellular signalling pathways including the phosphatidylinositol-3 kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (131, 132).

The IGFs are considered important regulators of pre-implantation conceptus development and placental development as well as normal growth (116, 117, 133-136), since both *IGF-I* and *IGF-II* knockout mice have birth weights that are 60% of normal control mice (136, 137). Null mice carrying mutations in both *IGF-I* and *IGF-II* die invariably after birth (136, 137). Deletion of IGF-I Type 1 receptor responsible for the actions of both IGF-I and IGF-II causes a greater degree of fetal growth retardation than deletion of either IGF suggesting that both ligands signal through IGF1R to promote growth in the fetus (137). In mice, IGF-II appears especially involved in the establishment of placental function because mice deficient in IGF-I have normal placental weights (137), whereas placental weights are markedly reduced in IGF-II deficient mice (135). During the preimplantation period, both *IGF-I* and *IGF-II* are expressed in the uterus, oviduct, ovary, and conceptus of domestic mammals, including cattle (138-142), sheep (116, 143-147), and pigs (148). *IGF-II* is an imprinted and paternally expressed gene in the fetus and placenta of mice, humans, and sheep (149), and plays an important role in regulation of fetal-placental growth/differentiation, stimulation of extravillous trophoblast migration/invasion, and facilitation of nutrient transfer through the development of placental exchange mechanisms (135, 150-156). In

mice, deletion of *IGF-II* in the labyrinthine trophoblast of the placenta reduced placental and fetal growth, and decreased passive permeability for placental exchange of nutrients (153). Consequently, alteration of *IGF-II* expression in human trophoblast during the first and early second trimester causes serious complications of pregnancy such as increased risk of intrauterine growth restriction, premature delivery, and preeclampsia. (157).

Although little is known of the roles of IGF-II in the peri-implantation ovine conceptus and endometrium, emerging evidence suggests that expression and differential actions with *IGF-I*, *IGF-II* and their receptor regulates epithelial-stroma interactions and endometrial gland morphogenesis to support uterine development (53, 158-160). Further IGF-II has been shown to affect expression of a number of nutrient transporters in human and murine placentae (161). In the *IGF-II* null placenta, system A amino acid transporters were decreased, while continuous expression of IGF-II increased amino acid and glucose transporters in the uterus. Therefore, IGF-II affects growth of fetal tissues not only directly as a mitogen, but also indirectly through changes in the placental environment that are necessary for delivery of nutrients to the fetus.

Secreted Phosphoprotein One (SPP1) During Pregnancy in Sheep

General structure of SPP1

SPP1, also known as osteopontin, is an acidic glycosylated phosphoprotein member of the small integrin binding ligand N-linked glycoprotein (SIBLING) family of proteins. Originally described in the mineralized matrix of bovine bone (162), SPP1 has since been detected in numerous epithelial cells and fluids (101-104). SPP1 contains a polyaspartic acid motif that can bind to hydroxyapatite and calcium ions and an RGD (Arg-Gly-Asp) amino acid sequence which binds to specific integrin heterodimers (163-165). Other characteristics of SPP1 include a hydrophobic leader sequence found in secreted proteins, a thrombin cleavage site and two glutamines that serve in transglutaminase-supported multimer formation (166). As a monomer of approximately 300 amino acids in length, SPP1 is subject to extensive post-translational modifications including phosphorylation, glycosylation and cleavage that results in various isoforms ranging in size from 25-75 kDa (167). Interestingly only ovine and bovine SPP1, but not

that of other species, has a 22 amino acid deletion that contains a potential calcium binding site and substitution of KS for RS at the thrombin cleavage site (166).

SPP1 interacts with $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, and $\alpha 8\beta 1$ via its (Arg-Gly-Asp (RGD) sequence to promote cell adhesion, spreading and migration. In addition to the RGD interactions, SPP1 mediates cell attachment through non-RGD mediated sites by its association with the $\alpha 4\beta 1$ integrin for leukocyte adhesion through an LDV (Leu-Asp-Val) peptide sequence (168). There is also a cryptic adhesive sequence recognized by $\alpha 9\beta 1$ (169). A secondary receptor for SPP1 is CD44 which is a hyaluronic acid receptor that mediates cellular responses similar to those of integrins including adhesion, migration, and proliferation (170, 171).

Function of SPP1 in the uterus

SPP1 affects a number of diverse processes such as bone mineralization, cancer metastasis, cell-mediated immune responses, inflammation, angiogenesis, and cell survival (164, 167). SPP1 has also been linked to the establishment and maintenance of pregnancy. As a major component of histotroph, SPP1 is recognized as the most highly upregulated protein during the early stages of pregnancy in uteri of humans and other mammalian species, including sheep (167, 172-177). SPP1 is capable of eliciting various effects throughout the body, through various proteolytic cleavage sites in the amino acid chain and through its integrin receptors (178). Multiple integrin receptors for SPP1 are present on trophoblasts and LE of humans and domestic animals, some of which increase during the peri-implantation period (179-182). Ovine and porcine trophoblast and LE cells show evidence of integrin receptor activation and cytoskeletal reorganization in response to SPP1 (181, 183). Further, polymerized SPP1 has high tensile strength when simultaneously binding receptors on different cells during adhesion and matrix assembly (184) which is supported by high levels of focal adhesion assembly at the maternal-conceptus interface (185). In the SPP1 knockout mouse (*Spp1*^{-/-}), there were no significant differences in the number of embryos carried by pregnant females, but the females had decreased pregnancy rates at mid-gestation and conceptuses were significantly smaller at all stages of gestation (186).

In sheep, SPP1 is synthesized and secreted during the peri-implantation period, a time when firm adhesion is developing between the uterine LE and conceptus (172, 187). SPP1 secreted by the uterine GE is progesterone regulated as administration of a PGR antagonist ablates *SPP1* mRNA expression (188). Among the isoforms, the 45 kDa form of SPP1 is more abundant in uterine flushings from pregnant than cyclic ewes (172, 189) and has greater binding affinity for $\alpha v \beta 3$ integrin than the native 70 kDa form (190).

In the process of implantation, SPP1 co-localizes with integrin $\alpha v \beta 3$ and plays a role in endometrial-embryonic signaling or facilitates embryonic attachment to uterine LE prior to invasion/attachment (179, 188, 191). SPP1 expression also increased in the caruncular component of placentomes, suggesting that in addition to progesterone, factors from the conceptus stimulate expression of SPP1 in uterine stroma that aid in vascularization at the maternal-conceptus interface (192). In the human endometrium, $\alpha v \beta 3$ integrin appeared abruptly on postovulatory Days 5 to 6 and maximal SPP1 expression (mRNA and protein) was observed during the mid- to -late secretory phase of the menstrual cycle (193). The co-localization of SPP1 with integrins at the apical surface of conceptus trophoblast and endometrial LE indicates that they may stimulate changes in proliferation, migration, survival, adhesion and remodeling of the conceptus during morphological changes and adherence to the uterine LE during implantation.

Amino Acids in Conceptus Development

Arginine

Arginine is the nitrogenous precursor for ornithine and nitric oxide (NO), a key signaling molecule with enormous physiological importance as it regulates vital metabolic pathways (194) such as implantation, embryogenesis, and uterine quiescence throughout gestation (91, 195-197). Arginine is one of the most abundant amino acids deposited in fetal tissue proteins and it increases in uterine fluid during the period of conceptus elongation (95, 198-200). In addition, supplementation of the diet with arginine increased embryonic and conceptus survival and growth rates in gilts and rats (201, 202). This evidence suggests that arginine is quantitatively important for fetal growth and development during pregnancy.

NO, a product of L-arginine catabolism, is essential to placental growth and angiogenesis for increasing uterine and placental-fetal blood flows (203). Polyamines are required for DNA and protein synthesis and for proliferation and differentiation of cells (204). In most animals, including sheep, synthesis of arginine occurs in enterocytes of the small intestine, which produce citrulline (the precursor of arginine) from glutamine and proline (205). However, this synthetic pathway is absent from uteri, placenta, and endometria of sheep (206). Limiting arginine or inhibiting NO synthesis resulted in increased fetal resorptions, intrauterine growth retardation (IUGR), increased perinatal mortality, and decreased number of live fetuses in pregnant rats (207). In contrast, intravenous administration of arginine enhanced fetal growth in an ovine model with IUGR (208). Similarly, dietary supplementation with arginine to gilts or rats during pregnancy increased the live-born litter weight (201, 202). Therefore arginine has an important role as a nutrient to the fetus that supports fetal survival and growth. Moreover, arginine is the critical regulator of nutrient utilization and protein synthesis through FRAP1 and NO signaling pathways (22, 96, 209).

Leucine

Since leucine is particularly important for mTOR signaling, and since blastocysts cultured *in vitro* consistently accumulate leucine and not other amino acids present in the culture media (96), it has been suggested that leucine uptake through transporters, such as sodium-dependent system SLC3A1 and sodium independent system L, stimulates mTOR signaling which triggers trophoblast motility and differentiation. This is supported by: 1) an increase in Na⁺ in uterine secretions at the time of implantation; 2) high expression of SLC3A1 at the blastocyst stage of development; 3) detection of SLC3A1 transport activity in porcine oocytes (96, 210); and 4) the presence of SLC7A5 and SLC7A8 across the trophoblast brush border of the pig placenta and sheep conceptus (211, 212). Leucine may also function to drive arginine transport as a counter transporter for uptake of arginine through the sodium-dependent system SLC3A1.

Glutamine

Glutamine is the physiological precursor of both ornithine and arginine in mammals, including pigs, sheep and rats (205, 213)(198). In particular, glutamine serves as an essential precursor for the synthesis of purine and pyrimidine nucleotides for cell division, amino sugars and NAD⁺ (nicotinamide adenine dinucleotide) (214)(199) and as an important energy source for rapidly dividing cells. In sheep, the maternal to fetal flux of glutamine was the greatest among all amino acids, especially between Day 13 and Day 16 pregnancy (95, 215) when conceptus growth is most rapid. The abundance of glutamine in fetal fluids is consistent with its important role in fetal nitrogen and carbon metabolism (216), as well as in early mammalian embryogenesis (217). Glutamate, a product of glutamine hydrolysis by glutaminase, binds with the N-methyl-D-aspartic acid receptor, resulting in an increase in the influx of calcium and its intracellular concentration and consequently activation of neuronal NOS (nNOS) for NO synthesis (218). Recently, Nicklin and coworkers (219) described a mechanism in which glutamine is required to facilitate the uptake of leucine, thereby leading to mTORC1 activation.

Glucose in Conceptus Development

Glucose, a major energy source for conceptuses (220), is delivered into the uterine lumen by glucose transporters (221, 222) and increases during pregnancy with associated conceptus elongation, especially in species with epitheliochorial and synepitheliochorial placentae (223). Transport of glucose from the maternal circulation into the uterine lumen is essential for pregnancy (224) as it can enhance rapid growth of conceptus trophectoderm by increasing cell proliferation through activation of the glutamine:fructose-6-phosphate amidotransferase (GFAT)-mediated MTOR signaling pathway (21). In pregnant pigs, total recoverable glucose in uterine flushings is greater than for cyclic pigs, but only increases after Day 12 of pregnancy in association with conceptus elongation (223). However, the presence and transport of glucose is related to a calcium spike and estrogen production by pig conceptuses that is coordinate with their survival and development (223). In ovine uterine flushings, total recoverable glucose increases 6-fold between Days 10 and 15 of gestation, but not the estrous cycle (95).

Transport of glucose across the cell membrane can be mediated by facilitative transporters (also known as GLUT) and/or sodium-dependent transporters (also known as SGLT) (225). Intensive studies on facilitative glucose transporters revealed that, of 13 family members, SLC2A1 to SLC2A12 and SLC2A13 are expressed in preimplantation blastocysts and conceptuses in mice, humans and cattle (222, 226, 227). Sodium-dependent glucose transporters, such as SLC5A1 and SLC5A11, transport glucose against electrochemical gradients, e.g., from endometrium into the uterine lumen (228). In sheep, *SLC2A1* and *SLC5A1* mRNAs and proteins were most abundant in uterine LE/sGE, whereas SLC2A4 was present in stromal cells and GE. *SLC5A11* mRNA was most abundant in endometrial GE. *SLC2A1*, *SLC2A3* and *SLC2A4*, *SLC5A1* and *SLC5A11* were expressed in trophectoderm and endoderm of conceptuses. Further, P4 induced and IFNT stimulated expression of *SLC2A1* and *SLC5A11*, indicating that facilitative and sodium-dependent glucose transporters both function in ovine uteri and conceptuses to transport glucose into the uterine lumen and for its uptake by the conceptus (94).

CHAPTER III
INSULIN-LIKE GROWTH FACTOR II ACTIVATES
PHOSPHATIDYLINOSITOL 3-KINASE-PROTOONCOGENIC PROTEIN
KINASE 1 AND MITOGEN-ACTIVATED PROTEIN KINASE CELL
SIGNALING PATHWAYS, AND STIMULATES MIGRATION OF OVINE
TROPHECTODERM CELLS

Introduction

Establishment and maintenance of pregnancy in eutherian mammals, including sheep, require reciprocal communication via endocrine and paracrine signals from the ovary, conceptus (embryo/fetus and associated extraembryonic membranes), and endometrium to support implantation and placentation. Implantation of the conceptus is an important and complex developmental event of early pregnancy and is a key evolutionary advance associated with viviparity (4, 41). During the peri-implantation period in the ovine uterus, the spherical blastocyst elongates to a tubular and then a filamentous form, and develops into a conceptus that then attaches to the uterine luminal wall. These events are supported by endometrial epithelial secretions and selective transport of molecules, including growth factors, cytokines, ions, glucose, and amino acids that are predominantly regulated by progesterone, and are required for peri-implantation conceptus survival, elongation, and development (4, 6, 24, 41, 61, 68, 76-78).

IGFs I and II are single chain polypeptides that are structurally similar to proinsulin (127, 229). The IGFs function as endocrine and paracrine/autocrine hormones that stimulate proliferation and differentiation in a variety of cell types, and mediate their effects by binding to specific cell membrane receptors, the type I and type II IGF receptors (IGF-IR and IGF-IIR, respectively) (230, 231). The IGFs are considered important regulators of pre-implantation conceptus development and placental development (116, 117, 133-136). During the preimplantation period, both *IGF-I* and *IGF-II* are expressed in the uterus, oviduct, ovary, and conceptus of domestic mammals, including cattle (138-142), sheep (116, 143-147), and pigs (148). *IGF-II* is an imprinted and paternally expressed gene in the fetus and placenta of mice, humans, and sheep (149),

and plays an important role in regulation of fetal-placental growth/differentiation, stimulation of extravillous trophoblast migration/invasion, and facilitation of nutrient transfer through the development of placental exchange mechanisms (135, 150-155). In mice, deletion of *IGF-II* in the labyrinthine trophoblast of the placenta reduced placental and fetal growth, and decreased passive permeability for placental exchange of nutrients (153). Furthermore, Smith *et al.* (157) reported that altered expression of *IGF-II* in human trophoblast during the first and early second trimester resulted in serious complications of pregnancy such as increased risk of intrauterine growth restriction, premature delivery, and preeclampsia.

Little is known of the biological roles of IGF-II in the peri-implantation ovine conceptus and endometrium. However, in the neonatal ovine uterus, expression of *IGF-I*, *IGF-II*, and *IGF-IR* regulates epithelial-stroma interactions and endometrial gland morphogenesis, suggesting that the ovine IGF system is important to support uterine development (158, 159). In sheep the IGF-IR is present in the pre-implantation blastocyst and in the luminal epithelium (LE), glandular epithelium (GE), and to a lesser extent, the caruncular stroma and myometrium of the uterus (144, 159, 160, 232). Specific cell signaling pathways stimulated by IGF-II in other cell types include activation of: 1) phosphatidylinositol 3-kinase (PI3K)/protooncogenic protein kinase 1 (AKT1); 2) FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), also known as mammalian target of rapamycin (mTOR); 3) ribosomal protein S6 kinase (RPS6K); 4) ERK1/2 (also known as p42/p44 or MAPK); and 5) P38 MAPK. However, these signaling pathways and their regulation by IGF-II in the ovine conceptus have not been investigated. Our working hypothesis is that IGF-II from the endometrium and within the conceptus regulates trophoblast growth and differentiation via the PI3K-AKT1 and MAPK signaling pathways. As a first step in testing this hypothesis, the present study was conducted to determine: 1) the distribution of *IGF-II* mRNA in ovine endometria and conceptuses during early pregnancy; 2) effects of IGF-II on PI3K-AKT1 and MAPK signaling pathways in mononuclear ovine trophectoderm (oTr) cells; and 3) effects of IGF-II on oTr cell migration.

Materials and Methods

Experimental design

Animals

Mature crossbred Suffolk ewes (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in experiments after they had exhibited at least two estrous cycles of normal duration (16–18 d). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Study 1

Tissue collection

At estrus (Day 0), ewes were mated to either an intact or vasectomized ram and then hysterectomized (n = 5 ewes per day) on either Day 10, 12, 14, or 16 of the estrous cycle, or Day 10, 12, 14, 16, 18, or 20 of pregnancy as described previously (62). At hysterectomy, the uterus was flushed with 20 ml 10mM Tris buffer (pH 7.0). Pregnancy was confirmed on Day 10–16 after mating by the presence of a morphologically normal conceptus(es) in the uterine flush. It was not possible to obtain uterine flushes on either Day 18 or 20 of pregnancy because the conceptus is firmly adhered to the endometrial LE. At hysterectomy, several sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the corpora lutea were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80 °C for subsequent protein extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the corpora lutea, and only tissues from the ipsilateral uterine horn were used in subsequent analyses.

Cloning of partial cDNA for ovine RPS6K

A partial cDNA for ovine *RPS6K* mRNA was amplified by RT-PCR using total RNA from Day 18 pregnant ovine endometrial tissues by specific primers based on data for the bovine *RPS6K* mRNA (GenBank accession no. AY396564; forward, 5'-ATT TGC CTC CCT ACC TCA CG-3'; reverse, 5'-AAT TTG ACT GGG CTG ACA GG-3'). PCR amplification was conducted as follows for ovine *RPS6K*: 1) 95 C for 5 min; 2) 95 C for 45 sec, 56.5 C for 1 min, and 72 C for 1 min for 35 cycles; and 3) 72 C for 10 min. The partial cDNAs for *RPS6K* were cloned into pCRII using a T/A Cloning Kit (Invitrogen Corp., Carlsbad, CA), and the sequence was verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (PerkinElmer Applied Biosystems, Foster City, CA).

In situ hybridization analyses

Location of mRNA expression in sections (5 μ m) of ovine uterine endometria and conceptuses was determined by radioactive *in situ* hybridization analysis as described previously (70). Briefly, deparaffinized, rehydrated, and deproteinized uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine *IGF-II* (39) and *RPS6K* partial cDNA using *in vitro* transcription with [α -³⁵S]uridine 5'-triphosphate. After hybridization, washing, and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), and exposed at 4 C for 6 days for *IGF-II* and 4 wk for *RPS6K*. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated through a graded series of alcohol to xylene, and coverslips affixed with Permount (Fisher Scientific). Images of representative fields were recorded under bright-field or dark-field illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

Immunohistochemistry

Immunocytochemical localization of phosphorylated (p)-AKT1, p-P38 MAPK, p-RPS6K, and p-ERK1/2 protein in ovine uteri and conceptuses was performed using

methods described previously (62). Rabbit antimouse phospho-AKT1 polyclonal IgG (Ser493/Thr308) (catalog no. 3061) at a 1:100 dilution, rabbit antihuman phospho-P38 MAPK monoclonal IgG (catalog no. 4631) at a 1:50 dilution, and rabbit antihuman phospho-RPS6K polyclonal IgG (Thr421/Ser424) (catalog no. 9204) at a 1:500 dilution were purchased from Cell Signaling Technology, Inc. (Danvers, MA), and rabbit antimouse phospho-ERK1/2 monoclonal IgG (catalog no. sc-7383) at 2.0 µg/ml was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antigen retrieval was performed using Pronase E digestion for p-AKT1, p-ERK1/2, p-RPS6K, and the boiling citrate method for p-P38 MAPK localization. Negative controls included substitution of the primary antibody with purified rabbit IgG at the same final concentration.

Study 2

Cell culture

Mononuclear oTr cells from Day 15 conceptuses were isolated and cultured as described previously (233). One cell line, referred to as oTr, was cultured in DMEM-F12 that included 10% fetal bovine serum, 50 U penicillin 50 µg streptomycin, 0.1 mm each nonessential amino acids, 1 mm sodium pyruvate, 2 mm glutamine, and 0.7 µM insulin. When the density of cells in dishes reached about 80% confluence, they were passaged at a ratio of 1:3, and frozen stocks of cells were prepared at each passage. For experiments, monolayer cultures of oTr cells (between passages 9 and 13) were grown in culture medium to 80% confluence on 100-mm tissue culture plates. Cells were serum and insulin starved for 24 h, and then treated with recombinant human IGF-II (50 ng/ml; R&D Systems, Inc., Minneapolis, MN) for 0, 15, 30, 60, and 90 min. Based on preliminary dose-response experiments, 50 ng/ml IGF-II was the dose selected for use in all experiments in the present study. This design was replicated in three independent experiments.

Western blot analyses

Whole cell extracts and immunoblot assays were prepared and performed as described previously (46). To harvest total cellular protein for Western blot analyses, oTr cells were rinsed with cold PBS and lysed by incubation in lysis buffer (1% Triton X-100,

0.5% Nonidet P-40, 150mmNaCl, 10mmTris, 1mmEDTA, 1mmEGTA, 0.2mmNa₃VO₄, 0.2 mm phenylmethylsulfonylfluoride, 50 mm NaF, 30 mm Na₄P₂O₇, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 30 min at 4°C. Cell lysates were passed through a 26-gauge needle and clarified by centrifugation (16,000 xg, 15 min, 4°C). The protein content was determined using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) with BSA as the standard. Proteins were denatured, separated using SDS-PAGE, transferred to nitrocellulose, and Western blot analyses were performed as described previously (43) using enhanced chemiluminescence detection (SuperSignal West Pico; Pierce, Rockford, IL) and X-OMAT AR x-ray film (Kodak) according to the manufacturer's recommendations. All antibodies used in these experiments were purchased from Cell Signaling Technology. Multiple exposures of each Western blot were performed to ensure linearity of chemiluminescent signals. Western blots were quantified by measuring the intensity of light emitted from correctly sized bands under UV light using a ChemiDoc EQ system and Quantity One software (Bio-Rad Laboratories).

Sandwich ELISA

Whole cell extracts were prepared as described previously (234, 235) and subjected to Sandwich ELISA according to the manufacturer's recommendations. Endogenous amounts of phospho-AKT1, phospho-P38 MAPK, and phospho-ERK1/2 protein were measured by Sandwich ELISA (catalog nos. 7160, 7140, and 7315, respectively) from Cell Signaling Technology.

Migration assay

Migration assays were conducted with oTr cells as described previously (236) with minor modifications. Briefly, oTr cells (50,000 cells per 100 µl serum and insulin-free DMEM) were seeded on 8-µm pore Transwell inserts (Corning Costar no. 3422; Corning, Inc., Corning, NY). Treatments were then added to each well (n = 3 wells per treatment). After 12 h, cells on the upper side of the inserts were removed with a cotton swab. For evaluation of cells that migrated onto the lower surface, inserts were fixed in 50% ethanol for 5 min. The transwell membranes were then removed, placed on a glass

slide with the side containing cells facing up, overlaid with Prolong antifade mounting reagent with 4',6- diamidino-2-phenylindole, and overlaid with a coverslip (Invitrogen-Molecular Probes, Eugene, OR). Migrated cells were counted systematically in five nonoverlapping locations, which covered approximately 70% of the insert membrane growth area, using a Zeiss Axioplan 2 fluorescence microscope with Axiocam HR digital camera and Axiovision 4.3 software (Carl Zeiss Microimaging, Thornwood, NY). The entire experiment was repeated at least three times with different batches of oTr cells between passages 7 and 10.

Statistical analyses

All quantitative data were subjected to least squares ANOVAs using the General Linear Model procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Western blot data were corrected for differences in sample loading using the α tubulin data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A *P* value less than or equal to 0.05 was considered significant. Data are presented as least-square means (LSMs) and their standard errors.

Results

IGF-II mRNA expression during the estrous cycle and pregnancy

IGF-II mRNA was abundant within caruncular stroma and, to a lesser extent, within the intercaruncular stratum compactum and stratum spongiosum stroma on Days 10 –12 of the estrous cycle and pregnancy (Fig. 3.1). By Day 14, for both cyclical and pregnant ewes, *IGF-II* mRNA was less abundant within the intercaruncular stroma, whereas *IGF-II* mRNA remained abundant within the endometrial caruncle. As ewes approached estrus on Day 16 of the estrous cycle, *IGF-II* mRNA was also localized to the LE in addition to the caruncular and intercaruncular stroma. Furthermore, *IGF-II* mRNA was more abundant within the myometrium on Day 16 of the cycle compared with any of the previous days of the estrous cycle and all days of pregnancy studied (data not shown). On Day 16 of pregnancy, *IGF-II* mRNA also increased in LE as noted for cyclical ewes; however, abundance of *IGF-II* mRNA within the caruncles declined

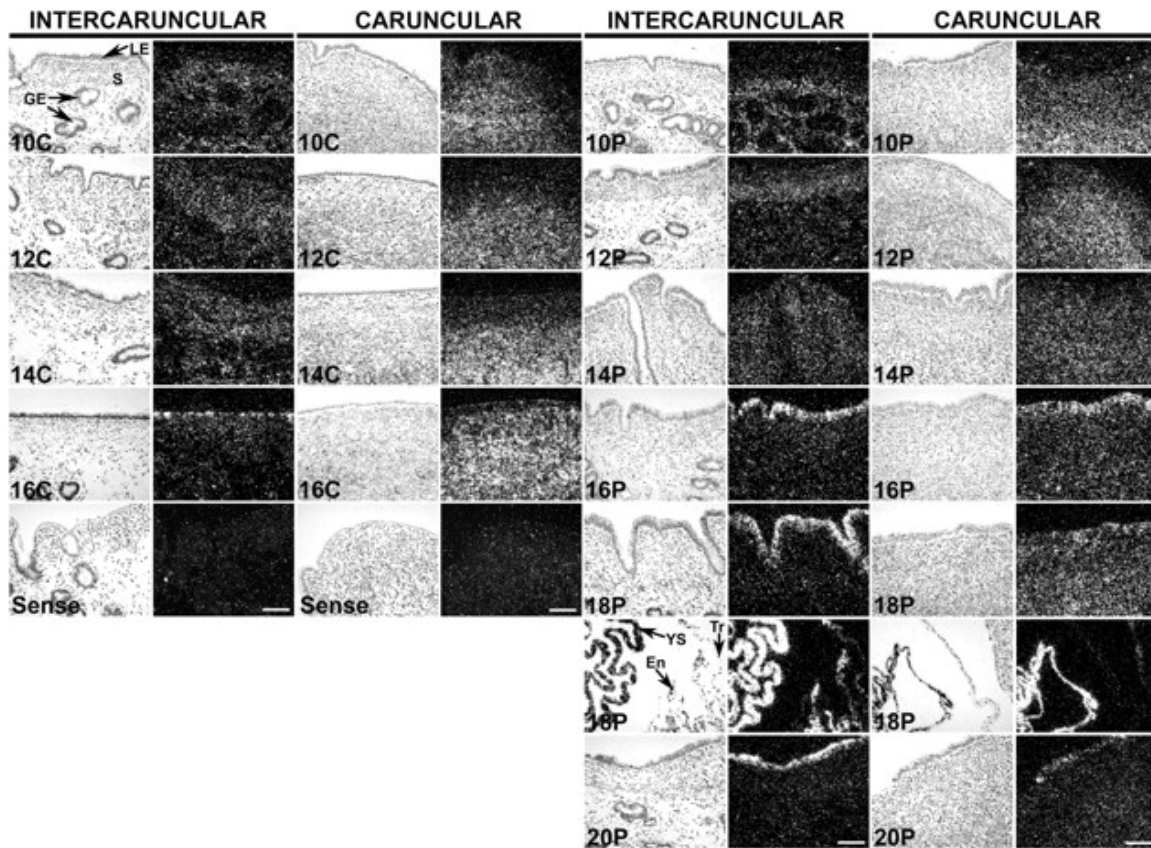


Fig. 3.1. *In situ* hybridization analyses of *IGF-II* mRNA in the uteri of cyclical and early pregnant ewes. Cross-sections of the uterine wall from cyclical (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IGF-II* cRNA probes. *IGF-II* mRNA was most abundant in compact stroma (S) of endometrial caruncles of both cyclical and pregnant ewes. Interestingly, *IGF-II* mRNA expression transitioned from the stroma to the luminal and superficial glandular epithelia in intercaruncular endometria between Days 15 and 20 of pregnancy. *IGF-II* mRNA was also present in all cells of the conceptus and particularly abundant in the yolk sac (YS) as shown for Day 18 of pregnancy. Scale bar represents 5 μ m. En, Endoderm; Tr, trophoderm.

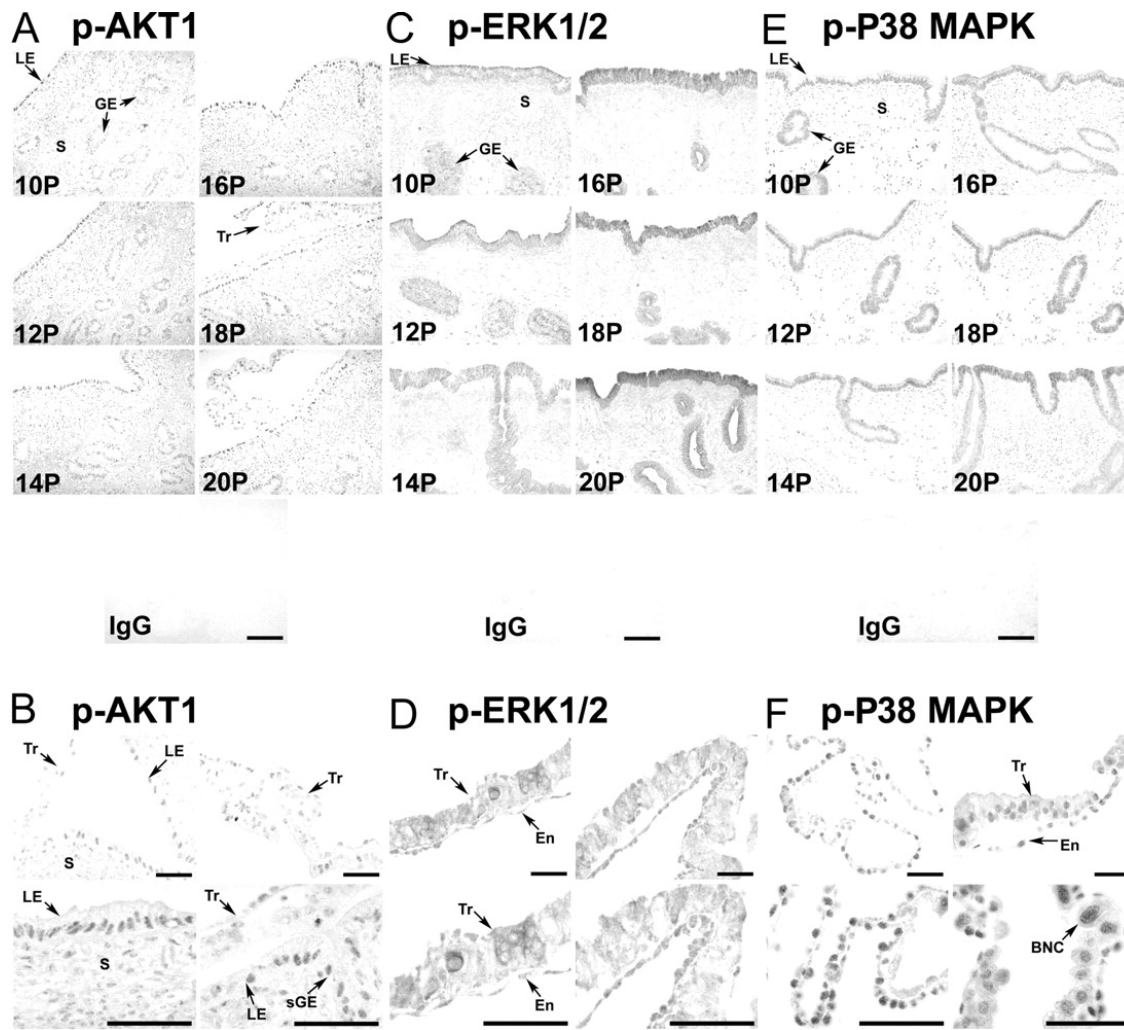


Fig. 3.2. Immunohistochemical localization of p-AKT1, p-ERK1/2, and p-P38 MAPK protein in endometria from pregnant (P) ewes from study 1. A, C, and E, Immunoreactive p-AKT1, p-ERK1/2, and p-P38 MAPK proteins, respectively, were localized using rabbit antimouse phospho-AKT1 polyclonal antibody (Ser493/Thr308), rabbit antimouse phospho-ERK1/2 monoclonal antibody, and rabbit antihuman phospho-P38 MAPK monoclonal antibody. Normal rabbit IgG was substituted for the primary antibody as a negative control. Sections were not counterstained. *Scale bar* represents 5 μm. B, D, and F, Immunoreactive p-AKT1, p-ERK1/2, and p-P38 MAPK proteins were present at low levels in most endometrial cells, but p-AKT1 and p-P38 MAPK were particularly abundant in the nuclei of endometrial LE and conceptus trophoderm (Tr) of pregnant ewes. Sections were not counterstained. *Scale bar* represents 2.5 μm. En, Endoderm; S, stroma.

compared with Day 16 of the cycle and previous days of pregnancy. *IGF-II* mRNA on Day 18 of pregnancy was also localized to LE and caruncular stroma, similar to Day 16 of pregnancy. *IGF-II* mRNA was particularly abundant in the yolk sac of the conceptus, but less abundant in the conceptus trophoctoderm and endoderm. By Day 20 of pregnancy, *IGF-II* mRNA in the caruncular stroma was low, whereas expression in LE was abundant, but variable in localization due to loss of the LE from fusion with trophoblast giant binucleate giant cells (BNCs). The conceptus endoderm, but not trophoctoderm, expressed abundant *IGF-II* mRNA on Day 20 of pregnancy.

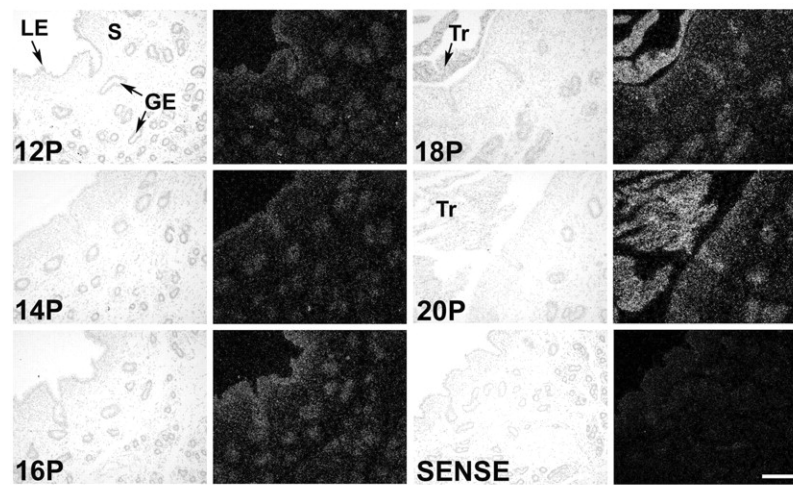
Localization of p-AKT1, p-ERK1/2, and p-P38 MAPK protein in ovine endometrium and conceptus

Immunohistochemical analyses revealed that p-AKT1, p-ERK1/2, and p-P38 MAPK proteins were present at low levels in most endometrial cells, but p-AKT1 and p-P38 MAPK were particularly abundant in the nuclei of endometrial LE and conceptus trophoctoderm in pregnant ewes. Localization of immunoreactive p-ERK1/2 and p-P38MAPK proteins in conceptus trophoctoderm, including BNCs, was based on cell morphology (Fig. 3.2).

Localization of RPS6K mRNA and p-RPS6K protein in ovine endometrium and conceptus

In situ hybridization analysis indicated that *RPS6K* mRNA was expressed mainly in endometrial LE and superficial ductal GE (sGE) (Fig. 3.3A). Furthermore, *RPS6K* mRNA was abundant in the conceptus trophoctoderm. Immunohistochemical analysis revealed that p-RPS6K protein was present at low levels in a majority of endometrial cells but was particularly abundant in the nuclei of endometrial LE and conceptus trophoctoderm of pregnant ewes (Fig. 3.3B).

A *RPS6K* mRNA



B *RPS6K*

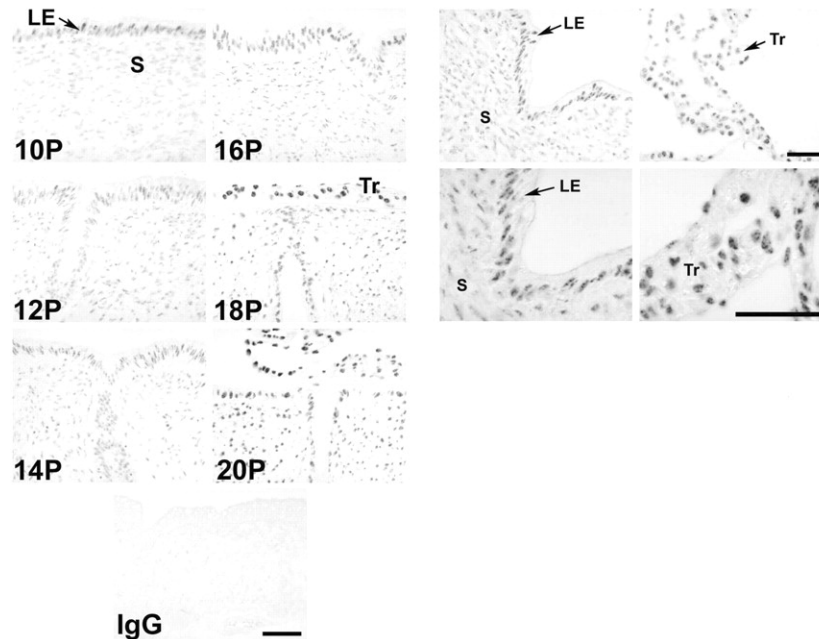


Fig. 3.3. *In situ* hybridization analysis of *RPS6K* mRNA and immunohistochemical analysis of RPS6K protein in the uteri of early pregnant ewes. A, Cross-sections of the uterine wall from pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *RPS6K* cRNA probes. *RPS6K* mRNA is expressed only in endometrial LE and sGE, and trophoblast (Tr) of the conceptuses. B, Immunoreactive p-RPS6K protein was localized using rabbit antihuman phospho-RPS6K (Thr421/Ser424) polyclonal antibody. Normal rabbit IgG was substituted for the primary antibody as a negative control. Sections were not counterstained. *Scale bar* represents 10 μ m (A) and 2.5 μ m (B). S, Stroma.

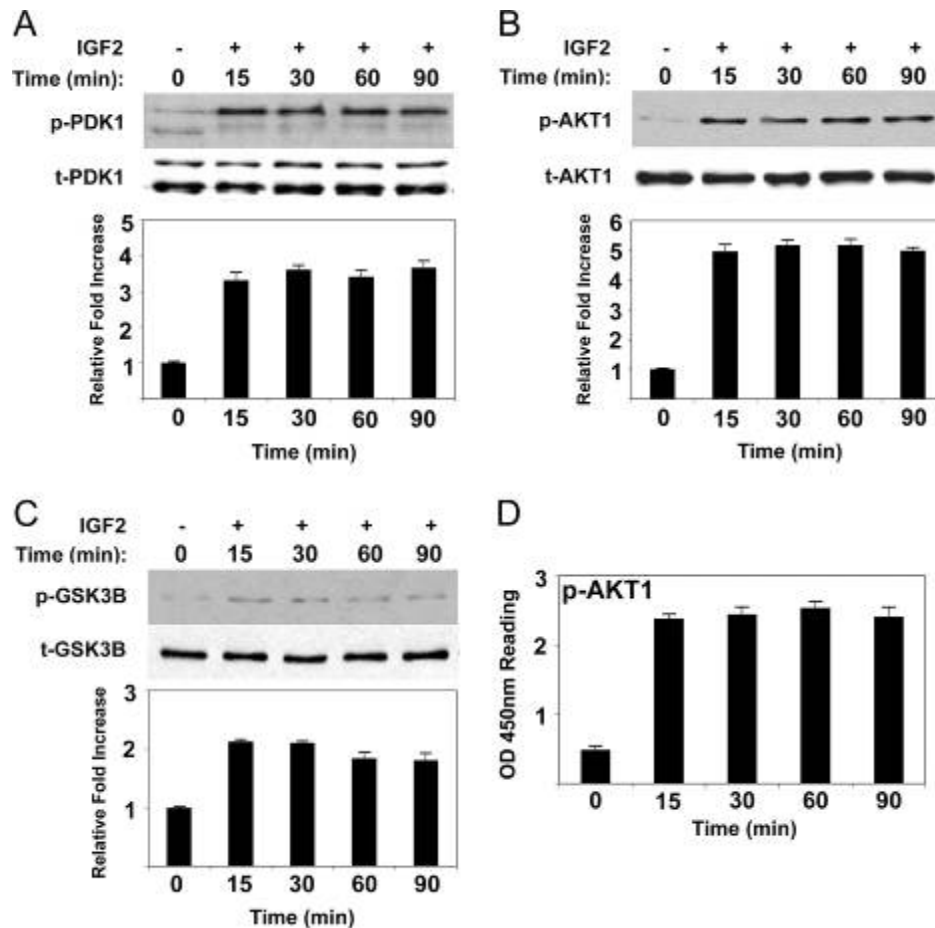


Fig. 3.4. IGF-II-induced phosphorylation of PDK1, AKT1, and GSK3B in oTr cells. Monolayers of 80% confluent oTr cells were serum starved for 24 h and then treated with IGF-II (50 ng/ml) for the indicated times. Blots were imaged to calculate the normalized values presented in *graphs (bottom)* by measurements of levels p-protein relative to total protein. The *bars* represent the relative abundance of p-protein. A, Phosphorylation of PDK1 by IGF-II. Representative Western blots for p-PDK1 (237) and total PDK1 (*middle*; visualized with total PDK1 antibody after stripping the same blot). IGF-II increased p-PDK1 level 2.5-fold ($P < 0.01$) over basal levels, within 15 min, and this activation was maintained to 90 min. B, Phosphorylation of AKT1 by IGF-II. Representative Western blots for p-AKT1 (237) and total AKT1 (*middle*; visualized with total AKT1 antibody after stripping the same blot). IGF-II increased p-AKT1 level 4-fold ($P < 0.001$) over basal levels within 15 min, and this activation was maintained to 90 min. C, Phosphorylation of GSK3B by IGF-II. Representative Western blots for p-GSK3B (237) and total GSK3B (*middle*; visualized with total GSK3B antibody after stripping the same blot). IGF-II increased p-GSK3B level 1.4-fold ($P < 0.05$) within 15 min, and this activation was sustained through 90 min. D, Measurement of endogenous levels of phospho-AKT1 protein in oTr cells using a Sandwich ELISA. Results of a Sandwich ELISA to determine activation of AKT1 ($P < 0.001$) were consistent with results from Western blot analyses. All quantitative data are presented as LSMs with overall standard errors of the means.

IGF-II activates PI3K-AKT1 and FRAP1-RPS6K signal transduction in ovine trophoblast cells

Western blot analyses of whole oTr cell extracts with antibodies to phosphorylated (p), i.e., activated target proteins, found that IGF-II increased levels of p-pyruvate dehydrogenase kinase (PDK) 1 and p-AKT1 by 2.5- ($P < 0.01$) and 4-fold ($P < 0.001$) over basal levels, respectively, within 15 min, and this activation was maintained to 90 min (Fig. 3.4A and B). In addition, results of a Sandwich ELISA to determine activation of AKT1 ($P < 0.001$) were consistent with results from Western blot analyses (Fig. 4D). IGF-II also stimulated a rapid 1.4-fold increase in p-glycogen synthase kinase (GSK) 3B ($P < 0.05$) within 15 min (Fig. 4C), as well as increases in p-FRAP1 ($P < 0.05$) and p-RPS6K (70/85 kDa) ($P < 0.001$) protein abundance between 0 and 15 min post-treatment that were sustained through 90 min (Fig. 3.5A and B). To determine the cell signaling pathways mediating effects of IGF-II on AKT1 and RPS6K, oTr cells were pretreated with pharmacological inhibitors of PI3K (200 nm wortmannin or 25 μ m LY294002) and FRAP1/mTOR kinase activity (50 nm rapamycin), respectively. Induction of p-AKT1 and RPS6K by IGF-II was inhibited by both PI3K inhibitors ($P < 0.01$ or $P < 0.001$), whereas rapamycin only inhibited RPS6K ($P < 0.01$) (Fig. 3.5C). These results suggested that activation of the PI3K-AKT1 pathway by IGF-II is required for translational activation of both p-FRAP1 and p-RPS6K in the IGF-II-induced cell signaling cascade.

IGF-II activates ERK1/2 and P38 MAPK phosphorylation in ovine trophoblast cells

Effects of IGF-II on MAPK signaling transduction cascades in oTr cells were evaluated on SDS-PAGE gel-separated and immunoblotted proteins probed with antibodies against p-P38 MAPK and p-ERK1/2 (p42/p44) proteins. In response to IGF-II, p-P38 MAPK increased 2.3-fold ($P < 0.01$) over basal levels within 30 min and decreased only slightly by 90 min (Fig. 3.6A). Meanwhile, IGF-II stimulated a rapid 2.6-fold ($P < 0.01$) increase in p-p44 (ERK1) and a 1.8-fold ($P < 0.05$) change in p-p42 (ERK2) within 15 min that was attenuated by 90 min (Fig. 3.6B). Results from the Sandwich ELISA verified that IGF-II activated p-P38 MAPK and p-ERK1/2 proteins within 15 min ($P < 0.05$) and that these proteins returned to basal levels at 90 min (Fig. 3.6 C and D).

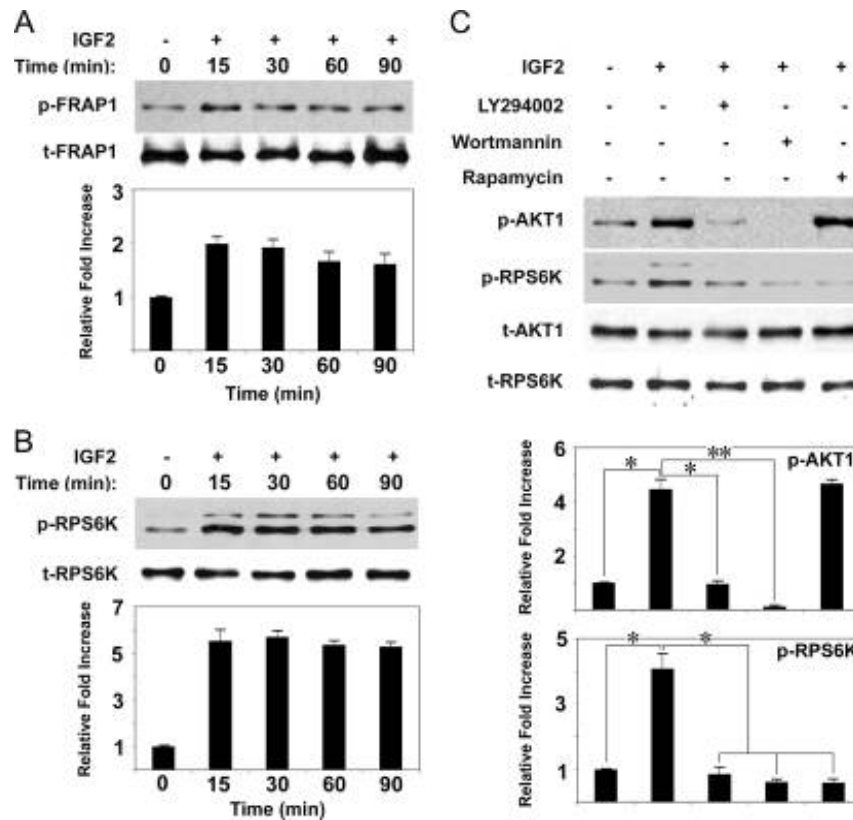


Fig. 3.5. IGF-II-induced phosphorylation of FRAP1 and RPS6K in oTr cells. Monolayers of 80% confluent oTr cells were serum starved for 24 h and then treated with IGF-II (50 ng/ml) for the times indicated. Blots were imaged to calculate the normalized values presented in the *graph (bottom)* by measurements of levels p-protein relative to total protein. The *bars* represent the relative abundance of p-protein. A, Phosphorylation of FRAP1 by IGF-II. Representative Western blots for p-FRAP1 (237) and total FRAP1 (*middle*; visualized with total FRAP1 antibody after stripping the same blot). IGF-II increased p-FRAP1 level 1.9-fold ($P < 0.05$) within 15 min, and this activation decreased about 1.7-fold by 90 min. B, Phosphorylation of RPS6K by IGF-II. Representative Western blots for p-RPS6K (237) and total RPS6K (*middle*; visualized with total RPS6K antibody after stripping the same blot). IGF-II increased p-RPS6K (70/85 kDa) level 1.4-fold ($P < 0.001$) within 15 min, and this activation was sustained through 90 min. C, Inhibition of AKT1 and RPS6K phosphorylation. Serum-starved oTr cells were pretreated with either 200 nm wortmannin, 25 μ M LY294002, or 50 nm rapamycin for 30 min and then stimulated with IGF-II (50 ng/ml) for 15 min. Representative Western blots for p-AKT1 (237), RPS6K (*middle*), total AKT1, and total RPS6K (*bottom*; visualized with total AKT1 and total RPS6K antibody after stripping the same blot) are presented. Blots were imaged to calculate the normalized values presented in the *graph* by measurements of levels p-AKT1 (238) and RPS6K (*lower*) relative to total AKT1 and total RPS6K. The *bars* represent the relative abundance of p-protein. Induction of p-AKT1 and RPS6K by IGF-II was inhibited by both PI3K inhibitors ($P < 0.01$ or $P < 0.001$), whereas rapamycin only inhibited RPS6K ($P < 0.01$). The *asterisk* (*) denotes an effect of treatment (* $P < 0.01$; ** $P < 0.001$). All quantitative data are presented as LSMs with overall standard errors of the mean.

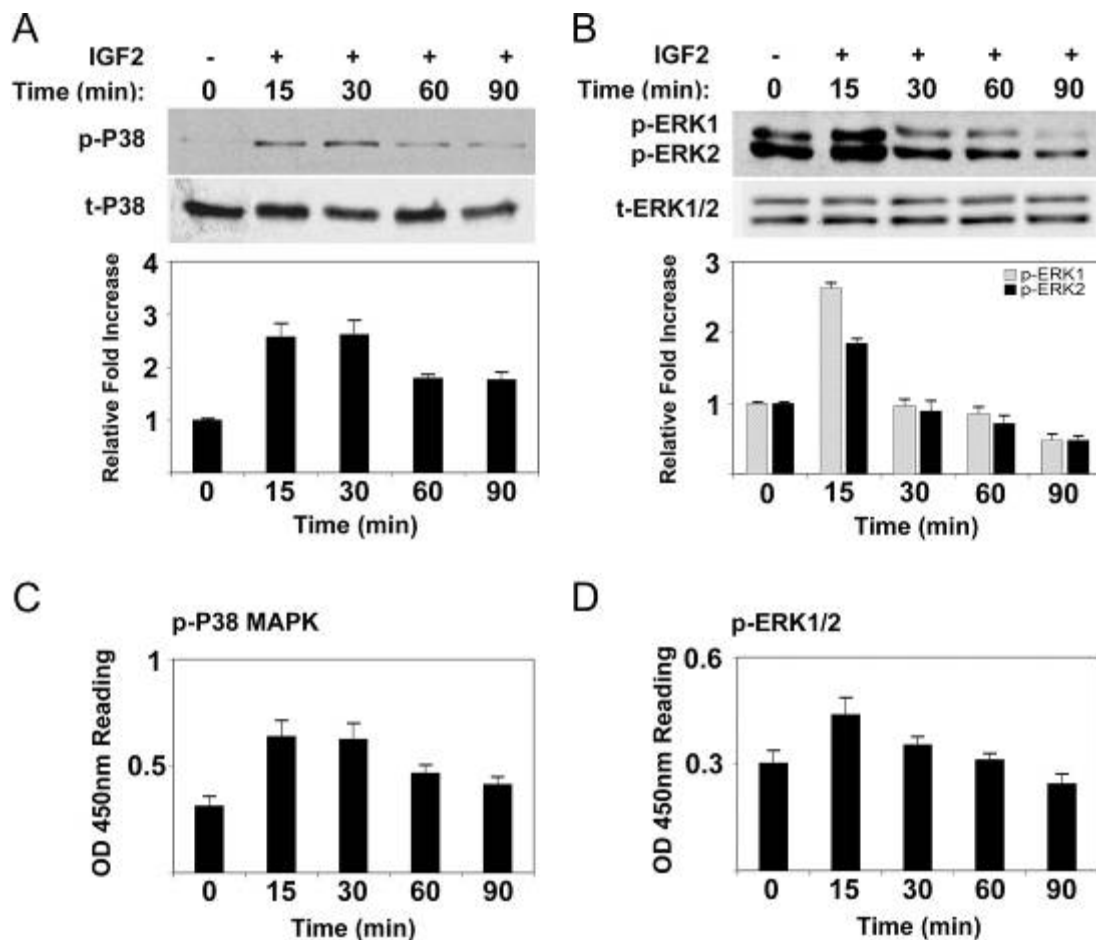


Fig. 3.6. IGF-II-induced phosphorylation of P38 MAPK and ERK1/2 in oTr cells. Monolayers of 80% confluent oTr cells were serum starved for 24 h and then treated with IGF-II (50 ng/ml) for the times indicated. Blots were imaged to calculate the normalized values presented in the *graph (bottom)* by measurements of levels p-protein relative to total protein. A, Phosphorylation of P38 MAPK by IGF-II. Representative Western blots for p-P38 MAPK (237) and total P38 MAPK (*middle*; visualized with total P38 MAPK antibody after stripping the same blot). IGF-II increased p-P38 MAPK level 2.3-fold ($P < 0.01$) within 30 min, and values decreased slightly by 90 min. B, Phosphorylation of ERK1/2 (p44/p42) induced by IGF-II. Representative Western blots for p-ERK1/2 (237) and total ERK1/2 (*middle*; visualized with total ERK1/2 antibody after stripping the same blot). IGF-II stimulated a rapid 2.6-fold ($P < 0.01$) increase in p-p44 (ERK1) and a 1.8-fold ($P < 0.05$) change in p-p42 (ERK2) within 15 min that was attenuated by 90 min. C, Measurement of endogenous levels of phospho-P38 MAPK protein in oTr cells using a Sandwich ELISA. D, Measurement of endogenous levels of phospho-ERK1/2 protein in oTr cells using a Sandwich ELISA. Results from the Sandwich ELISA verified that IGF-II activated p-P38 MAPK and p-ERK1/2 proteins within 15 min ($P < 0.05$), and that these proteins returned to basal levels at 90 min. All quantitative data are presented as LSMs with overall SE values.

IGF-II stimulates trophoderm cell migration

To investigate functional effects of IGF-II in oTr cells, cell migration assays were conducted. After longer incubation periods with IGF-II (12–48 h), amounts of total AKT1, P38 MAPK, and ERK1/2 (p42/p44) in oTr cells did not change (Fig. 3.7).

In cell migration assays (Fig. 3.8), treatment of oTr cells with IGF-II in serum and insulin-free DMEM increased their migration approximately 197%. Meanwhile, treatment of oTr cells with wortmannin, LY294002, and rapamycin along with IGF-II decreased oTr cell migration about 52, 73, and 46%, respectively. Of note, treatment of the oTr cells with the inhibitors alone for 12 h did not affect cell numbers (data not shown). Collectively, these results strongly support our hypothesis that IGF-II from endometrial LE acts in a paracrine manner on conceptus trophoderm to stimulate cell migration via activation of PI3K-AKT1 and MAPK signaling cascades.

Discussion

Ovine conceptuses undergo growth and morphological differentiation (transition from spherical to tubular to filamentous forms) between Days 13 and 14, immediately before attachment to the uterine epithelium (239). Factors affecting early placental development appear to have an important impact on the ability of the dam to supply sufficient nutrients for fetal growth because ovine placental growth occurs during the first half of pregnancy and reaches a plateau by Day 90 in advance of the period of rapid fetal growth and development (116). Among these factors, IGF-II is a recognized potent stimulator of cell proliferation, differentiation, and development, and it is considered to play a central role during implantation and establishment of pregnancy in most mammalian species, including humans, mice, and domestic animals (116, 140, 142, 148, 153, 157). IGFs play pivotal roles in fetal-placental development and may act, in part, to affect transfer of maternal nutrients to fetal-placental tissues throughout gestation (116, 142, 160). All components of the IGF system are present in the reproductive tract or placenta of ruminants at some stage of pregnancy (116). However, regardless of their spatial and temporal expression, mechanisms of action of IGFs have not been defined clearly. Results of the present study are the first to provide detailed analyses of temporal and spatial expression of *IGF-II* mRNA in both the ovine uterus and conceptus

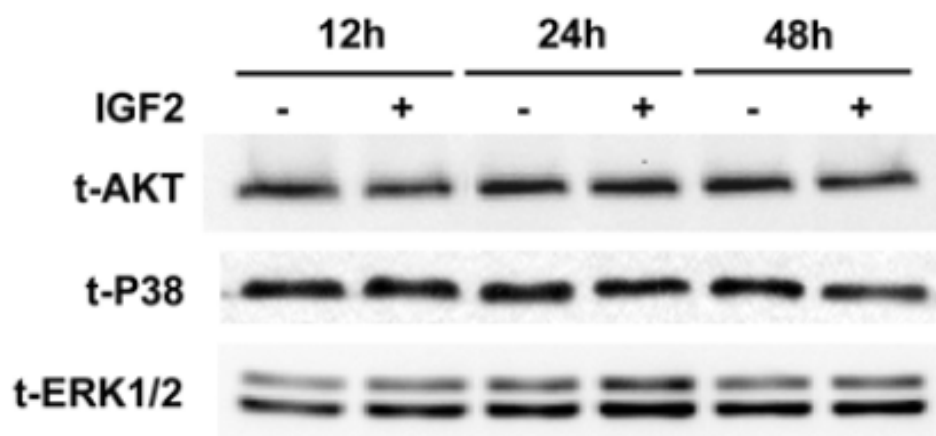


Fig. 3.7. Effects of IGF2 on total AKT1, P38 MAPK and ERK1/2 proteins in oTr cells. Monolayers of 80% confluent oTr cells were serum-starved for 24 h and then treated with IGF2 (50 ng/ml) for the indicated times. IGF2 did not change the abundance of AKT1, P38 MAPK or ERK1/2 (p42/p44) proteins in oTr cells.

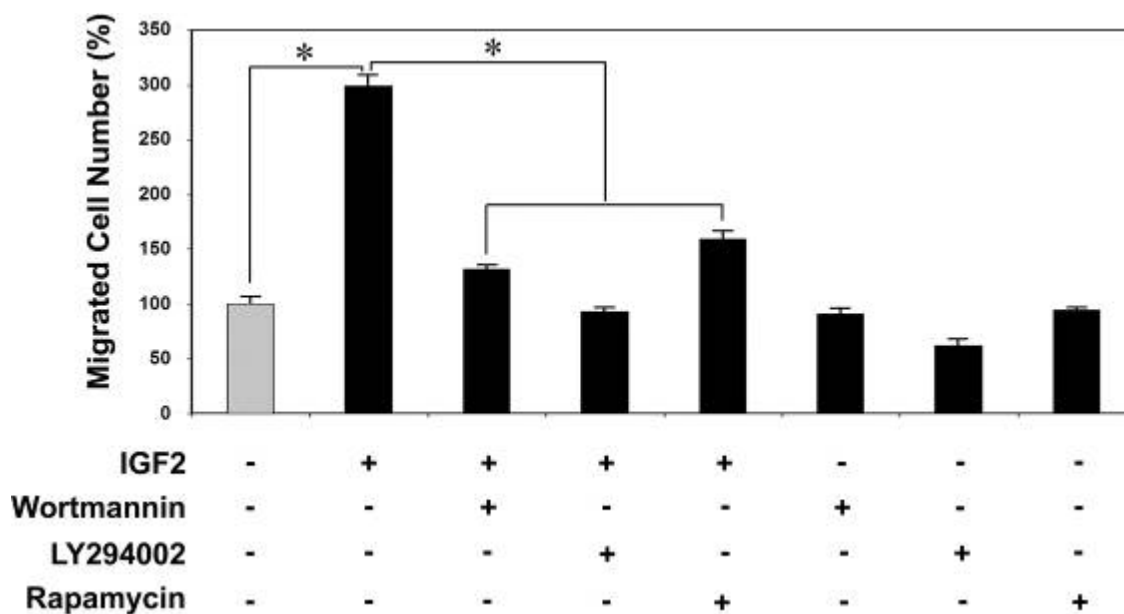


Fig. 3.8. IGF-II stimulates migration of oTr cells. The oTr cells were cultured in a transwell plate (n = 3 wells per treatment) and treated with recombinant human IGF-II, wortmannin, LY294002, rapamycin, or their combination. Cells grown in serum- and insulin-containing DMEM served as a negative control. Cell migration was determined after 12-h treatment and expressed as LSMs \pm SE. IGF-II stimulated ($P < 0.01$) and the inhibitors blocked ($P < 0.01$) the effect of IGF-II on cell migration. The *asterisk* (*) denotes an effect of treatment ($P < 0.01$).

throughout the estrous cycle and peri-implantation period of early pregnancy, as well as IGF-II-induced cell signaling pathways in oTr cells. The present results establish a direct link between IGF-II and cell signaling pathways (PI3K-AKT1-FRAP1-RPS6K, and/or ERK1/2 or P38 MAPK) which are clearly implicated in growth and differentiation of ovine conceptuses (Fig. 3.9).

In Study 1 *IGF-II* mRNA was most abundant in the compact stroma of endometrial caruncles, and was lower in the stroma of intercaruncular endometria of both cyclical and pregnant ewes (Fig. 3.1), as reported previously (144, 147). However, there was also abundant *IGF-II* mRNA in LE of intercaruncular endometria of Day 16 cyclic ewes. In human endometria, *IGF-II* mRNA is predominantly expressed in the mid- to -late secretory phase and is proposed to be a progestamedin (240-242). Notably, low levels of *IGF-II* mRNA are expressed in the rat and pig uterus, and does not exhibit distinct temporal or spatial (cell specific) changes during the estrous cycle (242-244). Interestingly, in the present study, *IGF-II* mRNA expression transitioned from stroma to LE in intercaruncular endometria between Days 15 and 20 of pregnancy, and was also expressed in all cells of the conceptus, but particularly abundant in the yolk sac. Similarly, in human and mouse, abundant *IGF-II* transcripts are found in primitive endoderm and extraembryonic mesoderm during early implantation (242, 245). It is known that the IGF-IIR acts as a signaling antagonist that prevents IGF-II responses by targeting IGF-II to lysosomes for degradation. For example, targeted disruption of the *IGF-IIR* gene in mice and studies with cultured rat granulosa cells showed that IGF-IIR is likely functioning as a clearance receptor for IGF-II (136, 246). Therefore, effects of IGF-II are considered to be mediated through the IGF-IR. Reynolds *et al.* (147) reported that *IGF-IR* mRNA is expressed by epithelial cells of superficial and deep uterine glands in ewes during early pregnancy. The IGF-IR was also detected in the trophectoderm cells of cotyledons of placentae of pregnant ewes using affinity cross-linking and immunohistochemical analyses (247). The spatial distribution of *IGF-II* mRNA in compact caruncular stroma and in LE of intercaruncular endometria supports our hypothesis that IGF-II plays essential roles in conceptus development and implantation in the ovine uterus, and that IGF-II mediated autocrine-paracrine cell signaling is essential for conceptus development.

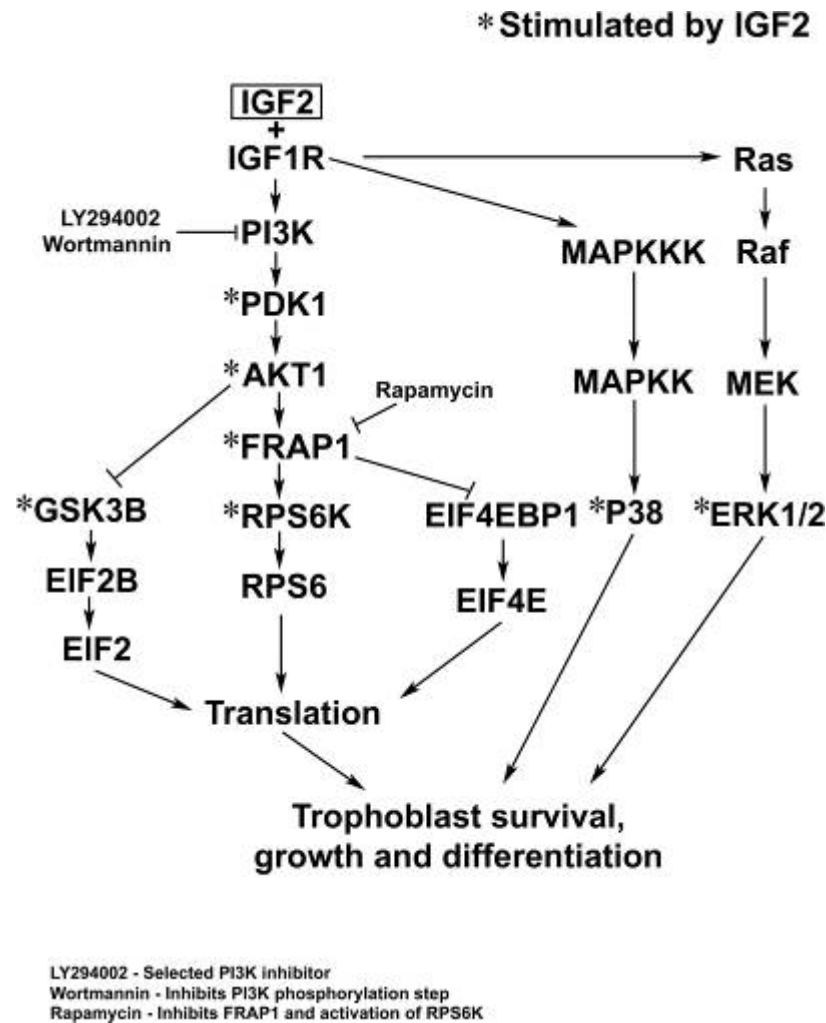


Fig. 3.9. Schematic illustrating the current working hypothesis on IGF-II-induced PI3K-AKT1-FRAP1-RPS6K and MAPK signaling cascades in oTr cells during the peri-implantation period. Evidence from the present study indicates that IGF-II stimulates multiple cell-signaling pathways noted by the *asterisks*. LY294002 and wortmannin are selective PI3K inhibitors. Rapamycin inhibits FRAP1. EIF2, eukaryotic translation initiation factor 2; EIF2B, eukaryotic translation initiation factor 2B; EIF4E, eukaryotic translation initiation factor 4ε; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; Ras, synaptic Ras-GTPase-activating protein.

The activities of both IGF-I and IGF-II are modulated by their association with members of a family of IGF binding proteins (IGFBPs) (231). By binding IGF-I and IGF-II, IGFBPs have growth-inhibitory effects by restricting availability of these ligands for binding to IGF-IR (231, 248). However, IGFBPs can also regulate IGF bioavailability by maintaining a circulating reservoir of IGFs and prolonging their half-life (249). IGFBPs synthesized in the uterus are considered to have important roles in implantation and placental development in many species (117, 244, 250). In the ovine uterus, *IGFBP-1* and *IGFBP-3* mRNAs were detected in the LE during early pregnancy (147, 251), *IGFBP-2*, *IGFBP-4*, and *IGFBP-6* mRNAs were expressed in dense caruncular stroma beneath overlying LE (147, 252), and *IGFBP-5* (253) mRNA was found in endometrial LE, GE, and stroma. Available evidence indicates that expression of IGFBPs is regulated by steroid hormones during the estrous cycle and also by conceptus factors during pregnancy (116, 242). Therefore, results of the present study and others indicate that ovarian factors (*e.g.* estrogen and progesterone) and conceptus-derived factors (*e.g.* IFNT) may act on ovine endometria to regulate *IGFBP* and *IGF-II* expression, as well as their interactions during implantation and placentation.

The PI3K-AKT1 cell signaling pathway has emerged as a critical component of insulin and IGF signaling cascades. Toyofuku *et al.* (254) demonstrated that IGF-I activates AKT1 via PI3K in human endometrium and decidua. However, to our knowledge, little is known of the signal transduction pathways activated by IGF-II in the conceptus during the peri-implantation period. In the present studies, oTr cells were used to demonstrate that IGF-II induces activation of PDK1-AKT1-GSK3B within 15 min and maintains activation to 90 min. Furthermore, IGF-II-induced phosphorylation of AKT1 was inhibited completely by wortmannin (PI3K-AKT1 inhibitor). In addition, immunoreactive p-AKT1 protein was present at low levels in most endometrial cells, but was particularly abundant in the nuclei of endometrial LE and conceptus trophoderm of early pregnant ewes. These results suggest that IGF-II plays an essential role in trophoblast survival, growth, and differentiation by activating the PI3K/AKT1 cell signaling pathway in the ovine uterus during the peri-implantation period.

Furthermore, IGF-II was found to induce phosphorylation of FRAP1-RPS6K in oTr cells. The FRAP1-RPS6K cell signaling cascade also mediates cell signaling by

nutrients such as amino acids and by mitogenic signals (255) to stimulate cell proliferation, differentiation, and gene expression. FRAP1, also known as mTOR, and PI3K-AKT1 pathways act in parallel to transduce growth factor signals and regulate common downstream targets such as RPS6K (242). In the present study, that low levels of *RPS6K* mRNA and protein were expressed in endometrial epithelia of pregnant ewes, while *RPS6K* mRNA and protein were most abundant in conceptus trophoctoderm during the periimplantation period. These results suggest that the FRAP1-RPS6K cell signaling cascade induced by IGF-II plays an important role in implantation events in the ovine uterus.

Results of the present study further demonstrated that IGF-II stimulates activation of ERK1/2 and P38 MAPK phosphorylation in oTr cells. As a family of protein kinases, MAPKs are highly conserved in most organisms, from yeast to humans (256). Among the three well-characterized subfamilies of MAPKs, the ERK1/2 and P38 MAPK pathways play important roles in differentiation processes, including embryonic and placental development (232, 257-259). However, little is known about either member of the MAPK subfamily in oTr development during early pregnancy. In the present study, immunoreactive ERK1/2 and P38 MAPK proteins were detected in ovine endometrial epithelia and conceptus trophoctoderm, including BNCs. Furthermore, IGF-II induced rapid increases in phosphorylation of both ERK1/2 and P38 MAPK, reaching a maximum at 15 or 30 min for oTr cells, respectively, followed by a decline through 90 min. Involvement of ERK1/2 and P38 MAPK in IGF-II-induced cell signaling in oTr cells indicates a novel cell signaling pathway. Therefore, IGF-II may influence ovine fetal/placental development by activating these MAPK pathways during the peri-implantation period.

In addition to activation of PI3K-AKT1 and MAPK cell signaling pathways, IGF-II increased migration of oTr cells, which was prevented by inhibitors of PI3K and FRAP1 (Fig.3.8). Thus, results of the present studies strongly support the hypothesis that IGF-II from the endometrial LE and/or conceptus tissues acts in a paracrine or autocrine manner to stimulate migration of conceptus trophoctoderm via activation of PI3K-AKT1 and MAPK signaling cascades.

In conclusion, IGF-II activates the PI3K-AKT1-FRAP1- RPS6K and stimulates the MAPK signal transduction cascades likely involved in expression of growth- and/or development-related genes affecting ovine fetal/placental cell migration during the peri-implantation period (Fig. 3.9). These results provide important insights into the mechanisms by which IGF-II regulates conceptus development during the peri-implantation period of sheep.

CHAPTER IV

SECRETED PHOSPHOPROTEIN 1 BINDS INTEGRINS TO INITIATE MULTIPLE CELL SIGNALING PATHWAYS, INCLUDING FRAP1/mTOR, TO SUPPORT ATTACHMENT AND FORCE-GENERATED MIGRATION OF TROPHECTODERM CELLS

Introduction

The mammalian target of rapamycin (mTOR/FRAP1) cell signaling pathway is an evolutionarily conserved serine/threonine kinase located downstream of phosphatidylinositol 3-kinase that controls cell growth and proliferation through regulation of protein synthesis (100, 260), as well as initiation of mRNA translation, ribosome synthesis, expression of metabolism-related genes, autophagy and cytoskeletal reorganization (102). mTOR is a “nutrient sensing system” that may be stimulated by molecules including insulin-like growth factor 2 (IGF2) and selected amino acids (22, 113, 114) to support blastocyst/conceptus (embryo and extra-embryonic membranes) development. Homozygous *Frap1* null mice die shortly after implantation due to impaired cell proliferation and hypertrophy in both the embryonic disc and trophoblast (12).

Cell attachment and migration are key events in implantation of blastocysts in uteri of all mammals. Trophoctoderm cells (Tr) of blastocysts attach to the uterine luminal epithelium (LE) for juxtaposition of conceptus and maternal circulations, leading to the establishment of a functional placenta. Attachment of Tr to LE is facilitated by a mosaic of interactions between integrins and extracellular matrix (ECM) proteins which contribute to stable adhesion at implantation (182, 261, 262). In sheep, conceptus elongation occurs prior to and is a prerequisite for the initiation of implantation and involves a rapid transition from spherical to tubular and filamentous forms between Days 10 and 15. Elongation of conceptuses involves extensive cytoskeletal reorganization in Tr cells to support their hypertrophy and migration (263). Implantation in sheep is noninvasive with increasing apposition and adhesion between Tr and uterine LE between Days 18 and 50 to 60 of gestation (24).

Secreted phosphoprotein 1 (SPP1/osteopontin), a multifunctional ECM protein, binds to cell surface integrin receptors to regulate basic cell-cell and cell-matrix interactions essential to cell adhesion, migration and proliferation (164, 167). SPP1 contributes to implantation in several mammalian species (167) and multiple integrin receptors for SPP1 are present on Tr and LE of humans and domestic animals, some of which increase during the peri-implantation period (167, 179, 182). Binding of SPP1 to integrins results in activation of integrin receptors and recruitment of cytoskeletal proteins to form focal adhesions in Tr cells (264), and *Spp1*^{-/-} and *Spp1*^{+/-} mice display decreased pregnancy rates at mid-gestation (186).

SPP1 induces motility in human trophoblast cells through mTOR signaling (265) and rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins stimulated by insulin-like growth factor (IGF1) such as focal adhesion kinase (FAK) (266). These results suggest a role for SPP1-induced mTOR complex signaling in key events of pregnancy. However, little is known about downstream transcription factor activation and transcription factor-mediated gene expression induced through SPP1-integrin interactions in mammalian Tr cells. Therefore, this study was designed to identify relationships and crosstalk between multiple membrane and intracellular cell signaling cascades activated by SPP1, including mTOR, and integrin binding to ovine Tr cells that control cell proliferation, migration, attachment and adhesion in conceptuses during the peri-implantation period of pregnancy. Results of these studies provide strong evidence for SPP1-induced integrin-mediated force-generated migration and cell adhesion mechanisms in Tr cells utilizing mTOR, MAPK14 (p38), MAPK1/MAPK3 (Erk1/2), as well as a novel link between p38 and mTOR signaling pathways.

Materials and Methods

Animals and tissue collection

All animal experiments complied with the Guide for Care and Use of Agricultural Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Ewes (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams. At estrus (Day 0) ewes were mated to rams and tissues were collected and prepared for analysis as previously described on either Day 16,

18 or 20 of pregnancy (n = 5 ewes/day) (267). Mononuclear ovine trophectoderm (oTr1) cells from Day 15 conceptuses were isolated and cultured as described previously (268). These cells constitute a relevant cell line for these studies because they exhibit numerous properties of ovine trophectoderm cells *in vivo*. Native SPP1 was isolated from bovine milk as described previously (269).

Integrin affinity chromatography and immunoprecipitation

Affinity chromatography experiments were performed as previously described (264). Bovine milk SPP1 (bSPP1) was coupled to cyanogen bromide-activated Sepharose 4B (Sigma, St. Louis, MO) at 1 mg/ml according to the manufacturer's instructions. Surface biotinylation of oTr1 cells was performed as previously described (270). Briefly, oTr1 cells were surface-labeled with biotin in 75 cm² flasks for 1 hour at room temperature and washed with PBS. Cells were lysed with 50 mM octyl- β -D-glucopyranoside (OG) (Fisher Scientific, Pittsburgh, PA) containing 1.5 mM each MnCl₂ and MgCl₂ for 30 min on an orbital shaker at 4°C. Cell extracts were centrifuged and then mixed at 20 min intervals with SPP1-Sepharose (0.5 ml) for 2 h at 0°C. The column was washed with 20 ml of 1% OG plus Mg²⁺ and Mn²⁺, and 0.5 ml fractions were eluted with 4 ml of 1% wt/vol OG + 10 mM EDTA. Thirty microliters of each fraction were separated on a 7% polyacrylamide gel under non-reducing conditions, transferred to polyvinylidene difluoride (PVDF) and blocked for 30 min with 5% wt/vol nonfat dry milk in Tris-buffered saline containing 0.1% vol/vol Tween 20. Blots were probed for biotin using streptavidin-alkaline phosphatase as previously described (168, 269). Integrins on oTr1 cells which bound to the SPP1-Sepharose column were identified by immunoprecipitation or Western blotting (264) using antibodies to human integrins including α v β 3 (clone LM609, Chemicon International, Temecula, CA), β 1 (clone DE9, Upstate), α v β 5 (clone P1F6, Chemicon), β 6 (clone CS β 6, Chemicon), α 4 (clone HP2/1, Abcam, Cambridge, MA), α 5 (AB1928, Chemicon), α v (AB1930, Chemicon), and β 3 (AB1968, Chemicon). Protein A-Sepharose (Pierce, Rockford, IL) was washed and suspended 1:1 with 0.5% vol/vol Triton X-100 in TBS. In 1.5 ml microcentrifuge tubes, 200 μ l of the bead mixture, 5 μ g of antibody, 150 μ l of pooled EDTA eluates and 600 μ l of 0.5% vol/vol Triton X-100 in TBS were combined. This mixture was rotated gently

overnight at 4°C, centrifuged briefly and washed 6 times with 1 ml of 0.5% vol/vol Triton X-100 in TBS. Beads were combined with 100 µl of 2X sample buffer and boiled for 10 min. Samples (30 µl) were separated by 7% SDS-PAGE under non-reducing conditions and transferred to PVDF. The blots were probed for biotin

To identify integrins that bound SPP1 directly, using Western blotting, 30 µl of labeled oTr1 cell lysate and pooled column eluates were separated by nonreducing SDS-PAGE and transferred to PVDF. The blots were blocked in 5% wt/vol nonfat dry milk for 1 h at room temperature and incubated overnight at 4°C with antibodies directed to $\alpha 5$ integrin (AB1928, Chemicon) or $\beta 1$ integrin (AB1952, Chemicon) subunits and visualized on autoradiography film using enhanced chemiluminescence (ECL).

Immunofluorescence microscopy for detection of integrin activation

The oTr1 cells were seeded onto bSPP1-coated Lab-Tek coverglass slides (Nalge Nunc International, Rochester, NY). After 2 h, cells were fixed with -20°C methanol and immunofluorescence staining was performed using an antibody to human integrin $\alpha \nu \beta 3$ (LM609, Chemicon) or human talin (TLN)(clone 8d4, Sigma) as previously described (181). Cells were then incubated with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (A11029, Invitrogen). Images were captured using a Zeiss Axioplan 2 microscope (Carl Zeiss) fitted with an AxioCam high-resolution digital camera using Axiovision 4.1 software. Alpha-actinin (ACTN) was localized in OCT-embedded uterine cross sections (Day 20 of pregnancy, n=4) as previously described (181). Tissue-bound ACTN antibody (A2543, Sigma) was detected with secondary antibody and slides were overlaid with DAPI before images were captured.

Activation of cell signaling intermediates

Effects of SPP1 on oTr1 cells were evaluated by cell culture and Western blot analyses (268). Briefly, oTr1 cells were cultured to 80% confluency, serum- and insulin-starved for 36 h and then treated with 100 ng/ml bSPP1 for 0, 15, 30, 60 or 90 min. For experiments utilizing inhibitors of cell signaling, including 50 nM rapamycin (mTORC1 inhibitor), 25 µM LY294002 (PI3K inhibitor), 25 µM PD098059 (MEK inhibitor), 10 µM SB203580 (inhibitor of p38) and 10 µM blebbistatin (inhibitor of myosin II)

incubation was for 60 min. Cell extracts were separated using 8, 10 or 12% SDS-PAGE and then cell signaling intermediates were detected using the antibodies and visualized using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL). Correctly-sized bands were quantified using a ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA). All inhibitors were tested for cytotoxicity by MTT analysis (271). Immunocytochemical localization of p-RPS6 protein in ovine uteri and conceptuses was performed using boiling citrate buffer for antigen retrieval and methods described previously (268). Immunoprecipitation was performed on whole cell extracts after antibodies to P70S6K or p38 were added to extracts and bound proteins were purified using Protein A/G Plus agarose (234). Proteins liberated from beads were separated by SDS-PAGE and analyzed by Western blotting.

Effects of bSPP1 on p-p38 were evaluated using Sandwich ELISA. Whole cell extracts were prepared as described previously (234) and subjected to Sandwich ELISA (Cell Signaling Technology, 7140) according to the manufacturer's recommendations.

Cell proliferation assay

Effects of SPP1 on proliferation of oTr1 cells seeded at 50% confluency in polystyrene microwells (Corning-Costar) were evaluated following treatment with soluble bSPP1 or BSA at 10, 100 or 1000 ng/ml (n=3 replicates/treatment) for 48 h. Cells were fixed in formalin, stained with Amido black, solubilized with 2N NaOH and absorbance readings were taken at 595 nm.

Cell migration assay

Effects of SPP1 on oTr1 cell migration were evaluated as previously described (264, 268). oTr1 cells were seeded in a confluent layer 8 μ m pore transwell inserts (Corning-Costar, Corning, NY) after the bottom side of each filter was coated with 100 μ g/ml bovine serum albumin (BSA). bSPP1 or BSA were then added to lower wells in serum-free DMEM-F12 at concentrations of 10, 100 or 1000 ng/ml (n=3 replicates/treatment). After 12 h, cells remaining on the top portion of the membrane were removed by scraping with a cotton swab and membranes were fixed in 4% wt/vol paraformaldehyde (PAF) for 5 min. Membranes were removed, placed on slides and

stained with 4',6'-diamidino-2-phenylindole (DAPI)(P36935, Invitrogen). Cells that migrated to the bottom surface of the membrane were counted in five independent sections of each membrane, which accounted for approximately 70% of the membrane area, using a Zeiss Axioplan 2 fluorescence microscope with an AxioCam HR digital camera and Axiovision 4.3 software.

Additional cell migration assays were performed with the addition of the inhibitors to cell signaling, either alone or in combination (see results). The oTr1 cells were also seeded onto Four-well LabTek chambered slides coated with bSPP1 and cultured for 1 h with 0, 10, 25 or 50 μ M blebbistatin to determine effects of SPP1 to promote focal adhesion-mediated cell spreading.

Cell adhesion assays

Effects of SPP1 on oTr1 cell adhesion were evaluated as previously described (168). Polystyrene microwells (Corning-Costar) were coated overnight at 4°C with two-fold serial dilutions (20 μ g/ml to 20pg/ml) of the following proteins (50 μ l) in phosphate-buffered saline (PBS) (n=3 replicates/treatment): bSPP1, rSPP1 (RGD), rSPP1 (RAD), hFN (positive control) or BSA (negative control). After blocking each well in 10 mg/ml BSA in PBS (100 μ l), 50,000 oTr1 cells were added per well and allowed to attach for 1 h (37°C, 5% CO₂). In all cell attachment experiments, nonadherent cells were removed by washing in isotonic saline and wells were fixed in 10% vol/vol formalin in PBS. Plates were stained with 0.1% wt/vol Amido black for 15 min, rinsed and solubilized with 2 N NaOH to obtain an absorbance reading at 595 nm which directly correlated with the number of cells stained in each well (272). Additional attachment assays were performed in which cation levels were varied to determine whether attachment of oTr1 cells to SPP1 was integrin-dependent (168). The experiment was performed and attachment quantified as described previously, but in the presence or absence of divalent cations (2 mM Ca²⁺, 1 mM Mg²⁺) in cation-free Puck's Saline A. Experiments to block integrin-mediated oTr1 cell adhesion (273) were conducted as described previously except that cells were pre-incubated for 15 min with blocking antibodies (25 μ g/ml) to either integrin α v β 3 (LM609, Chemicon), α 5 (P1D6, Chemicon) or α 2 β 1 (BHA2.1, Chemicon).

In vitro bead assay for activation of focal adhesions at apical membranes

In vitro bead assays were performed as previously described (264). Briefly, polystyrene beads (10 μm ; Polysciences Inc., Warrington, PA) coated with either rSPP1(RGD) or rSPP1(RAD) were allowed to settle onto oTr1 cells seeded onto Lab-Tek coverglass chamber slides coated with collagen Type I. After 1 h cells were fixed in 2% paraformaldehyde, permeabilized in 1% Triton X-100 and immunofluorescence co-localization was used to detect integrin αv (AB1930, Chemicon) and TLN (264). Optical slice images from the basal to apical plasma membrane of oTr1 cells were captured using a Zeiss Stallion Dual Detector Imaging System with Intelligent Imaging Innovations Software (Carl Zeiss Inc., Thornwood, NY). Fluorescence deconvolution of images was used to compare and verify integrin activation, cytoskeletal reorganization and focal adhesion assembly at the basal and apical aspects of oTr1 cells.

Statistical analyses

All quantitative data were subjected to least squares analyses of variance using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Western blot data were corrected for differences in sample loading using the TUBA data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P-value ≤ 0.05 was considered significant. Data are presented as least-square means (LSM) with standard errors (SE).

Results

SPP1 binds $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ integrin heterodimers to activate focal adhesion assembly at the basal surface of trophectoderm cells

Immunoprecipitations of integrins in oTr1 cell lysates were performed using antibodies directed to integrin subunits reported to be expressed on ovine Tr cells including $\alpha 4$, $\alpha 5$, αv , $\beta 1$, $\beta 3$, $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ (Fig. 4.1A, first panel). Affinity chromatography was used to evaluate integrin expression on oTr1 cells. Detergent extracts of surface-biotinylated oTr1 cells were incubated with SPP1 from bovine milk

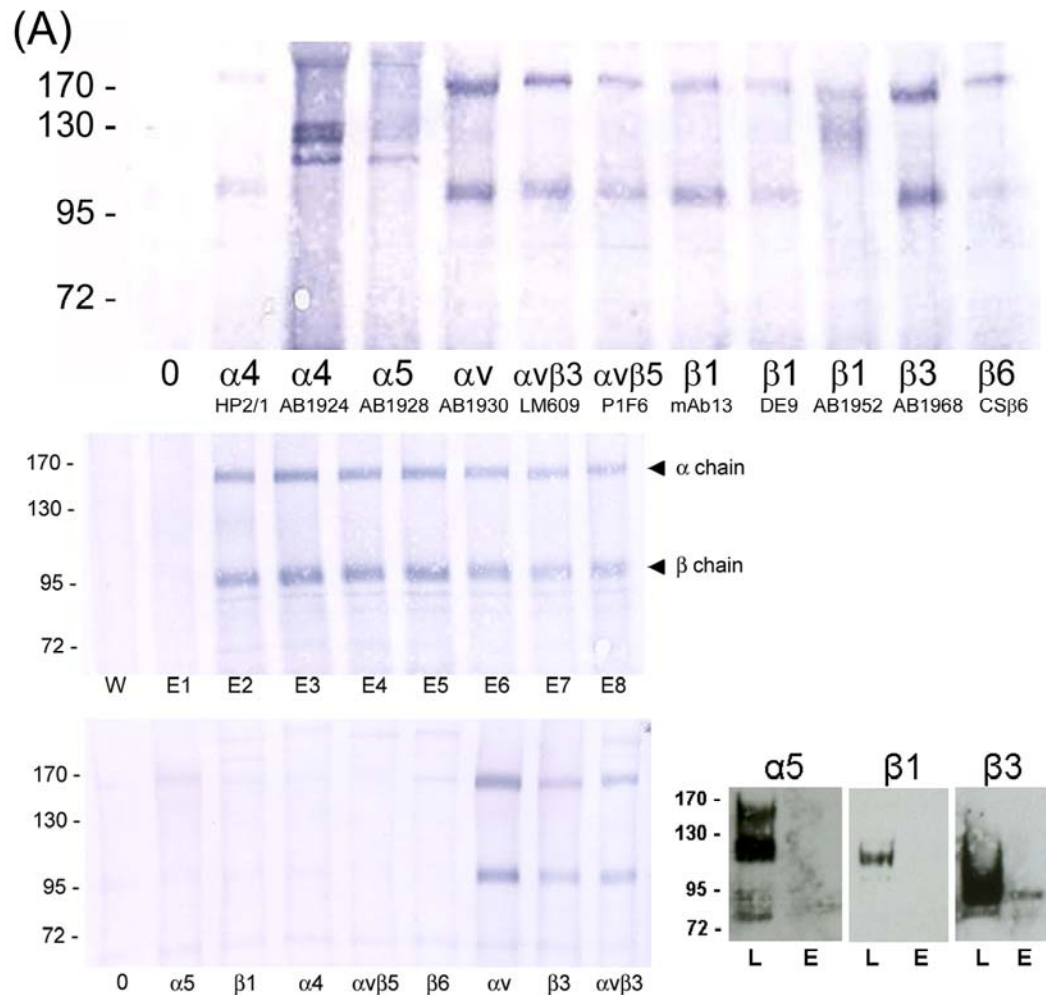


Fig. 4.1. SPP1 binds $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrin heterodimers to activate focal adhesion assembly at the basal surface of oTr1 cells. Panel A, First Panel: Streptavidin was used to detect biotinylated proteins in oTr1 cell lysates immunoprecipitated using antibodies to various integrins. Panel A, Second Panel: Proteins that bound to an SPP1-Sepharose column and were eluted with EDTA. 0 = no antibody. Panel A, Third Panel: Proteins that bound to an SPP1-Sepharose column, eluted with EDTA and immunoprecipitated using antibodies to various integrins. 0 = no antibody. Panel A, Fourth Panel: Western blot demonstrating the presence of $\alpha 5$ and $\beta 1$ integrin subunits in oTr1 cell lysates (L), but not in SPP1-Sepharose column EDTA eluates (E). The $\beta 3$ integrin subunit was used as a positive control. Panel B, Immunofluorescence detection of $\alpha v\beta 3$ integrin heterodimer and talin (TLN) at the basal surface of cells cultured on SPP1. Mouse IgG (mIgG) served as a negative control for integrin $\alpha v\beta 3$ and TLN. Panel C, Immunofluorescence detection of $\alpha 5$ and $\beta 1$ integrin subunits at the basal surface of cells cultured on SPP1 and of talin (TLN) or $\beta 1$ integrin subunit at the basal surface of cells cultured on type I collagen. Mouse IgG or rabbit IgG served as negative controls. Panel D, Immunofluorescence detection of ACTN at the interface of trophectoderm (Tr) and uterine luminal epithelium (LE) on Day 20 of pregnancy in ewes. Mouse IgG served as a negative control.

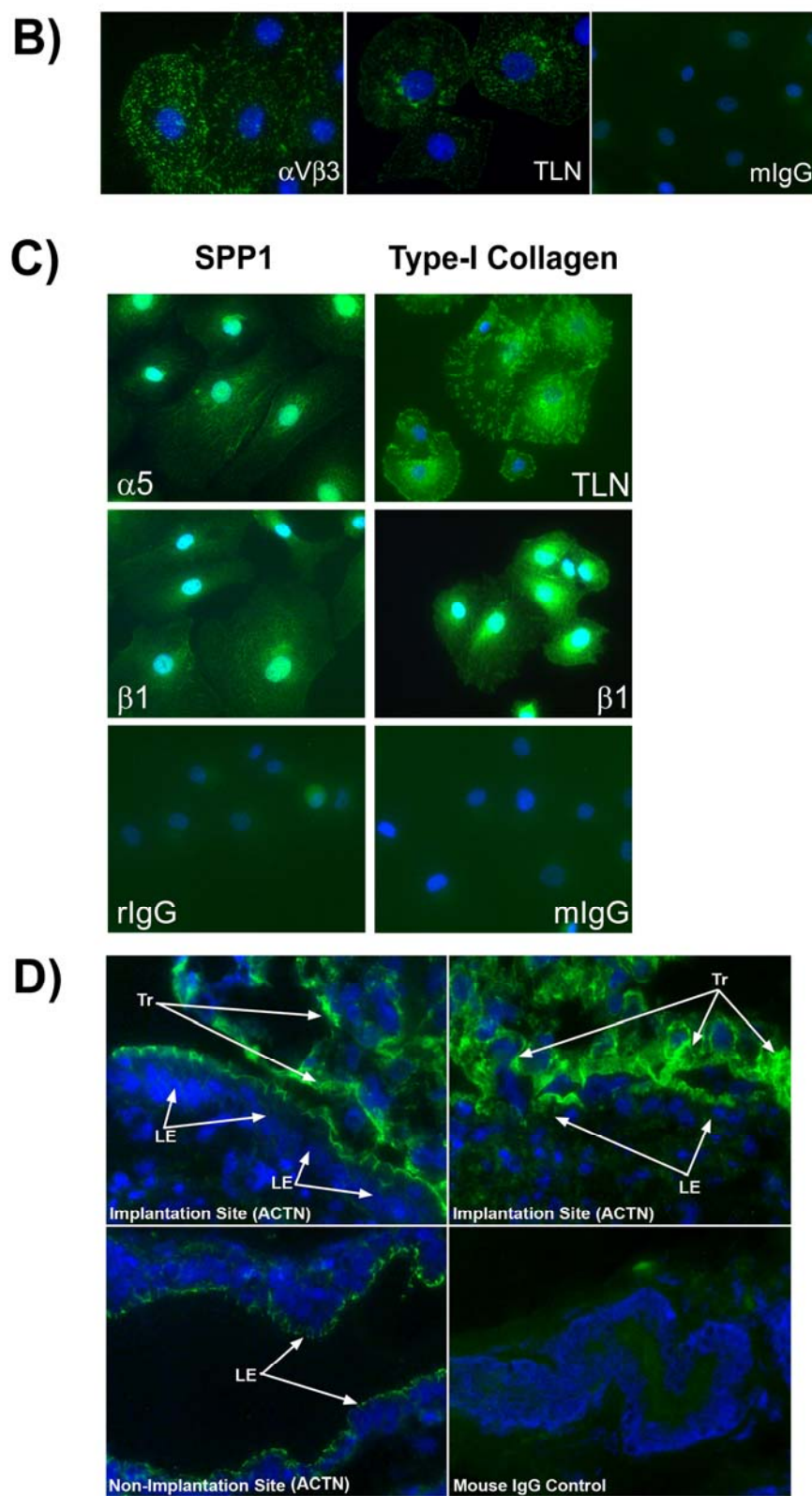


Fig. 4.1 continued.

(bSPP1)-Sepharose in the presence of 1.5 mM Mg^{2+} and 1.5 mM Mn^{2+} . After incubation, the column was washed in the presence of 1.5 mM Mg^{2+} and 1.5 mM Mn^{2+} and eluted with EDTA to chelate cations and release bound integrins. Integrins eluted in fractions E2, E3 and E4, with α -integrin chains migrating at approximately 170 kDa and β -integrin chains migrating at 100-120 kDa (Fig. 4.1A, second panel).

Successful immunoprecipitation of labeled oTr1 integrins occurred with antibodies to αv , $\beta 3$ and $\alpha 5$ integrin subunits, as well as an antibody to the integrin $\alpha v\beta 3$ heterodimer (Fig. 4.1A, third panel; note that the bands detected migrated at the same sizes as the bands in the eluates shown in the second panel). An antibody to the αv integrin subunit also precipitated a β chain, presumed to be the $\beta 3$ integrin subunit, as an antibody to the $\beta 3$ integrin subunit precipitated an α chain at the same relative size as the bands precipitated by an antibody to the $\alpha v\beta 3$ heterodimer. Thus, the $\alpha v\beta 3$ integrin on oTr1 cells binds SPP1.

We were unable to immunoprecipitate the $\alpha 5$ or $\beta 1$ integrin subunits from membrane extracts of biotinylated oTr1 cells that were eluted from an SPP1-Sepharose column or identify $\alpha 5$ or $\beta 1$ integrin subunits from the same eluates by Western blotting (Fig. 4.1A, fourth panel). However, based upon the $\alpha 5$ integrin subunit immunoprecipitation results from labeled oTr1 cells, the fact that the $\beta 1$ subunit is the only known binding partner for $\alpha 5$, as well as the complexity of $\alpha 5\beta 1$ integrin binding to ECM, immunofluorescence studies were performed to evaluate the impact of functional binding of $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins at the basal surface of oTr1 cells.

Because $\beta 1$ integrins, but not the $\alpha v\beta 3$ heterodimer, bind collagen (274), slides were coated with bSPP1 or collagen Type I and immunostained using antisera to $\alpha v\beta 3$, $\alpha 5$, $\beta 1$ or talin (TLN). The $\alpha v\beta 3$ integrin heterodimer and TLN were detected as large aggregates at the basal surface of oTr1 cells on slides coated with bSPP1 (Fig. 4.1B). The $\beta 1$ integrin subunit was also detected in small aggregates at the basal surface of oTr1 cells on slides coated with bSPP1 and larger $\beta 1$ integrin aggregates were detected in cells grown on collagen (Figure 4.1C). These results suggest that the $\alpha v\beta 3$ and possibly $\alpha 5\beta 1$ integrins functionally aggregate at the basal surface of oTr1 cells in response to bSPP1 to elicit focal adhesion assembly and integrin activation during ovine Tr cell attachment to immobilized bSPP1.

The $\alpha 5$ and $\beta 1$ aggregates on the basal surface of cells grown on slides coated with SPP1 are intriguing and may provide insight into the function and binding of this integrin in Tr during pregnancy. Unlike $\alpha v\beta 3$ which binds to the RGD sequence in numerous ECM proteins, $\alpha 5\beta 1$ integrin binding to ECM appears to be restricted to fibronectin (FN) and secreted phosphoprotein 1 (SPP1) (274). Further, the binding of both SPP1 and FN to $\alpha 5\beta 1$ is more complex than the interaction between SPP1 and other integrin receptors. For instance, adhesion of SPP1 to the $\alpha v\beta 3$ integrin is not affected by alteration of SPP1 structure at, or adjacent to, the integrin recognition sequence. In contrast, binding of SPP1 and FN to $\alpha 5\beta 1$ requires not only binding to the RGD portion of the integrin recognition sequence, but also binding to both a synergy site (the PHSRN sequence in the FN III₉ repeat for FN or the SVVYGLR site for SPP1) and a heparin binding site (275). Both FN and SPP1 have a heparin binding site which, in FN, is required for focal adhesion formation (276). The heparin binding site in SPP1 overlaps with the thrombin cleavage site and likely represses SPP1 proteolysis (277). It is also noteworthy that the N-terminal fragment generated by thrombin cleavage containing both the RGD and SVVYGLR sequences is a stronger ligand for the $\alpha 5\beta 1$ integrin than full length SPP1 (275). Therefore, for optimal SPP1 binding to $\alpha 5\beta 1$, the proper presentation of the protein is required, and this presentation is likely different when the protein is coated on the rigid surface of a glass slide than when it is linked to a Sepharose bead. It is possible that the $\alpha 5\beta 1$ integrin isolated in a membrane extract will not bind efficiently to SPP1 without the availability of a synergy site and a heparin binding site. It is also likely that the SPP1 on beads remains in the intact full-length form, whereas it is quite conceivable that SPP1 at the base of cultured cells is converted into its thrombin cleavage fragment by serine proteases secreted by the oTr1 cells themselves. Interestingly, cleavage of SPP1 occurs *in vivo* within the lumen of pregnant animals; the source of enzymes responsible for this process may be elongating conceptus trophoctoderm (183, 187). We hypothesize that our immunofluorescence data demonstrating aggregation of $\alpha 5$ and $\beta 1$ integrin subunits into focal adhesions at the basal surface of oTr1 cells on SPP1-coated glass contain the unique complement of binding sites to utilize the $\alpha 5\beta 1$ integrin to bind to SPP1 in contrast to the $\alpha 5\beta 1$ in membrane extracts attached to Sepharose beads. While we cannot definitively state that SPP1 binds $\alpha 5\beta 1$ integrin on

oTr1 cells due to the chromatography results, this possibility can not be excluded due to the focal adhesion assembly at the base of oTr1 cells in culture. Indeed the more physiological configuration of the SPP1 in the latter case suggests that SPP1 likely binds the $\alpha 5\beta 1$ integrin on oTr1 cells.

To establish the ability of ovine Tr cells to undergo integrin activation and assemble focal adhesions *in vivo* during implantation, cryosections of Day 20 ovine implantation sites containing conceptus Tr cells attached to uterine LE cells were also subjected to immunofluorescence analyses. Alpha-actinin (ACTN) was detected in aggregates within conceptus Tr at implantation sites (Figure 1D). ACTN is an actin binding and bundling protein associated with the cytoplasmic tail of beta integrin subunits in focal adhesions, as well as the zonula adherens (adherens junctions). ACTN became increasingly distributed across the apical surfaces of uterine LE and conceptus Tr cells on Day 20 of pregnancy, whereas no changes in ACTN distribution were associated with adherens junctions in adjacent uterine glandular epithelia within or outside implantation sites. This indicates recruitment of ACTN to the apical surface of uterine LE and conceptus Tr during assembly of focal adhesions.

SPP1 activates PI3K-AKT1 and FRAP1-P70S6K-RPS6 signal transduction in trophectoderm cells

The oTr1 cells were serum-starved for 48h, incubated in the presence or absence of bSPP1 and subjected to protein extraction. Based on dose-response experiments (Fig. 4.2A), bSPP1 was used at 100 ng/ml in experiments to determine cell signaling pathways mediating effects of bSPP1 on P70S6K. Western blot analyses of whole oTr1 cell extracts with antibodies to phosphorylated target proteins indicated that bSPP1 did not affect levels of phospho-PDK1 (p-PDK1), but increased phospho-mTOR (p-mTOR) and phospho-AKT1 (p-AKT1) and by 1.2- ($P<0.05$) and 1.7-fold ($P<0.05$) over basal levels, respectively, within 60 min and this effect was maintained to 90 min (Fig. 4.2B-D). bSPP1 also stimulated a rapid 2.7-fold increase in p-P70S6K ($P<0.01$) protein within 30 min post-treatment that was sustained through 60 min; levels of p-P70S6K then decreased at 90 min compared to 60 min, but were still higher than basal levels (Fig. 4.2E). The oTr1 cells were pretreated with pharmacologic inhibitors of PI3K (10 μ M

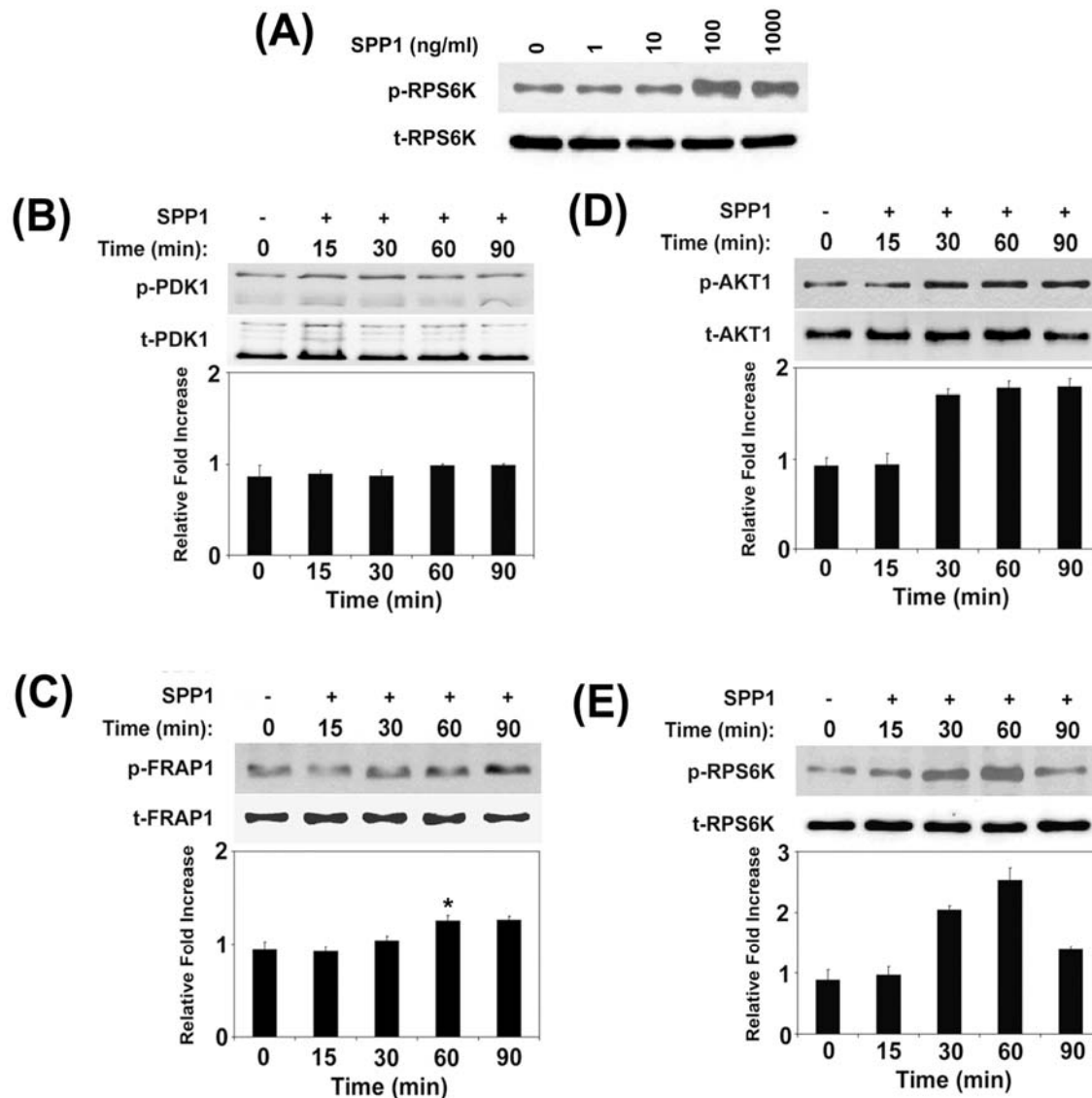


Fig. 4.2. SPP1 activates mTOR, P70S6K and RPS6 signal transduction in oTr1 cells. Western blot analyses of various intracellular signaling intermediates in oTr1 cells treated with soluble SPP1: A, Detection of p-RPS6K following treatment with increasing doses of bSPP1; B, Detection of p-PDK1; C, Detection of p-mTOR; D, Detection of p-AKT1; E, Detection of p-P70S6K. F, Detection of p-P70S6K following pharmacological inhibition of intracellular signaling with LY294002 and/or rapamycin; G, Detection of p-RPS6 following treatment with soluble SPP1; H, Immunohistochemical detection of p-RPS6 in conceptus trophoctoderm at implantation sites on Days 18 and 20 of pregnancy in ewes. Asterisks indicate statistically significant differences among treatments ($P < 0.05$).

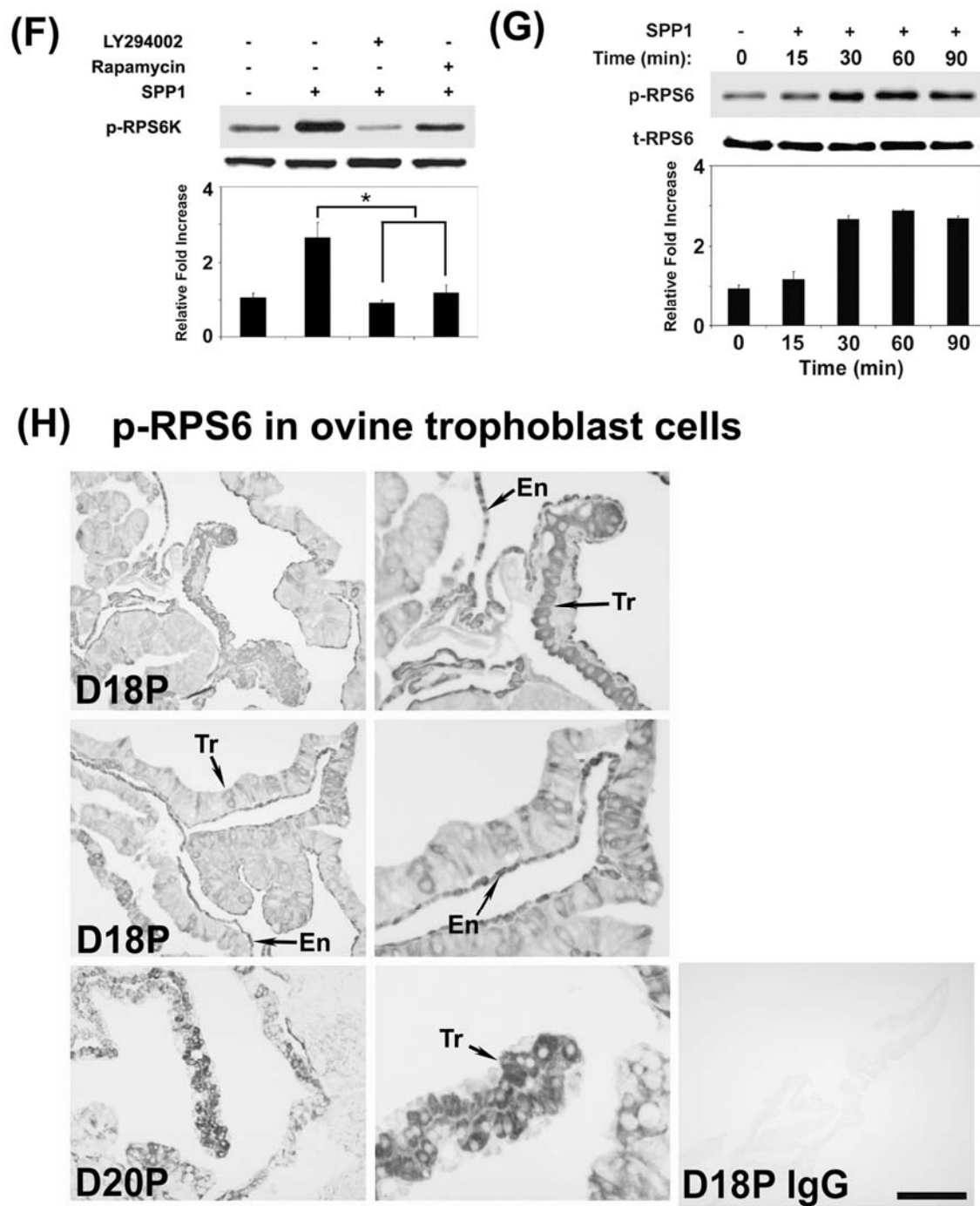


Fig. 4.2 continued.

LY294002) and mTOR kinase activity (25 nM rapamycin), respectively. Induction of phosphorylated P70S6K by bSPP1 was inhibited by both PI3K and mTOR inhibitors ($P<0.05$) (Fig. 4.2F). In addition, bSPP1 stimulated an increase in p-RPS6, a ribosomal protein phosphorylated by P70S6K, by 2.9-fold ($P<0.01$) within 30 min and this effect was maintained to 90 min (Fig. 4.2G). Immunohistochemical analysis revealed that p-RPS6 protein was highly expressed in the cytoplasm of conceptus Tr cells from uterine implantation sites on Days 18 and 20 of pregnancy (Fig. 4.2H). These results demonstrated that bSPP1 activates PI3K-AKT1 cell signaling to stimulate the mTOR-P70S6K-RPS6 cell signaling cascade in oTr cells.

SPP1 activates p38 and Erk1/2 phosphorylation in trophectoderm cells

Phosphorylation of p38 and Erk1/2 in response to bSPP1 in whole oTr1 cell extracts was examined using antibodies against p-p38 and p-Erk1/2 proteins. In response to 100 ng/ml bSPP1, p-p38 increased 2.9-fold ($P<0.05$) within 60 min and the effect was maintained to 90 min (Fig. 4.3A). In support of these results, sandwich ELISA detected increases in p-p38 protein within 30 min of bSPP1 treatment that was maintained through 90 min ($P<0.05$) (data not shown). bSPP1 also stimulated a 1.7-fold ($P<0.05$) increase in p-Erk1/2 within 60 min that was maintained to 90 min (Fig. 4.3B).

To determine the cell signaling pathways mediating effects of bSPP1 on p38 and Erk1/2, oTr1 cells were pretreated with pharmacologic inhibitors of MAPK-kinase (MEK) (25 μ M PD098059) and p38 (10 μ M SB203580). The bSPP1-induced increase in p-p38 was inhibited specifically by SB203580 ($P<0.05$) (Fig. 4.3C) and bSPP1-induced activation of Erk1/2 was specifically inhibited by PD098059 ($P<0.001$) (Fig. 4.3D). Collectively, these results revealed that bSPP1 activates the p38 and Erk1/2 cell signaling pathways in oTr1 cells.

SPP1 activated p38 mediates P70S6K signaling in trophectoderm cells (crosstalk between MAPK and mTOR intracellular signaling)

MAPK has been functionally linked to mTOR cell signaling (278); therefore, we determined effects of bSPP1 on interactions between P70S6K and p38 in the mTOR/P70S6K and MAPK signaling cascades using oTr1 cells pretreated with bSPP1

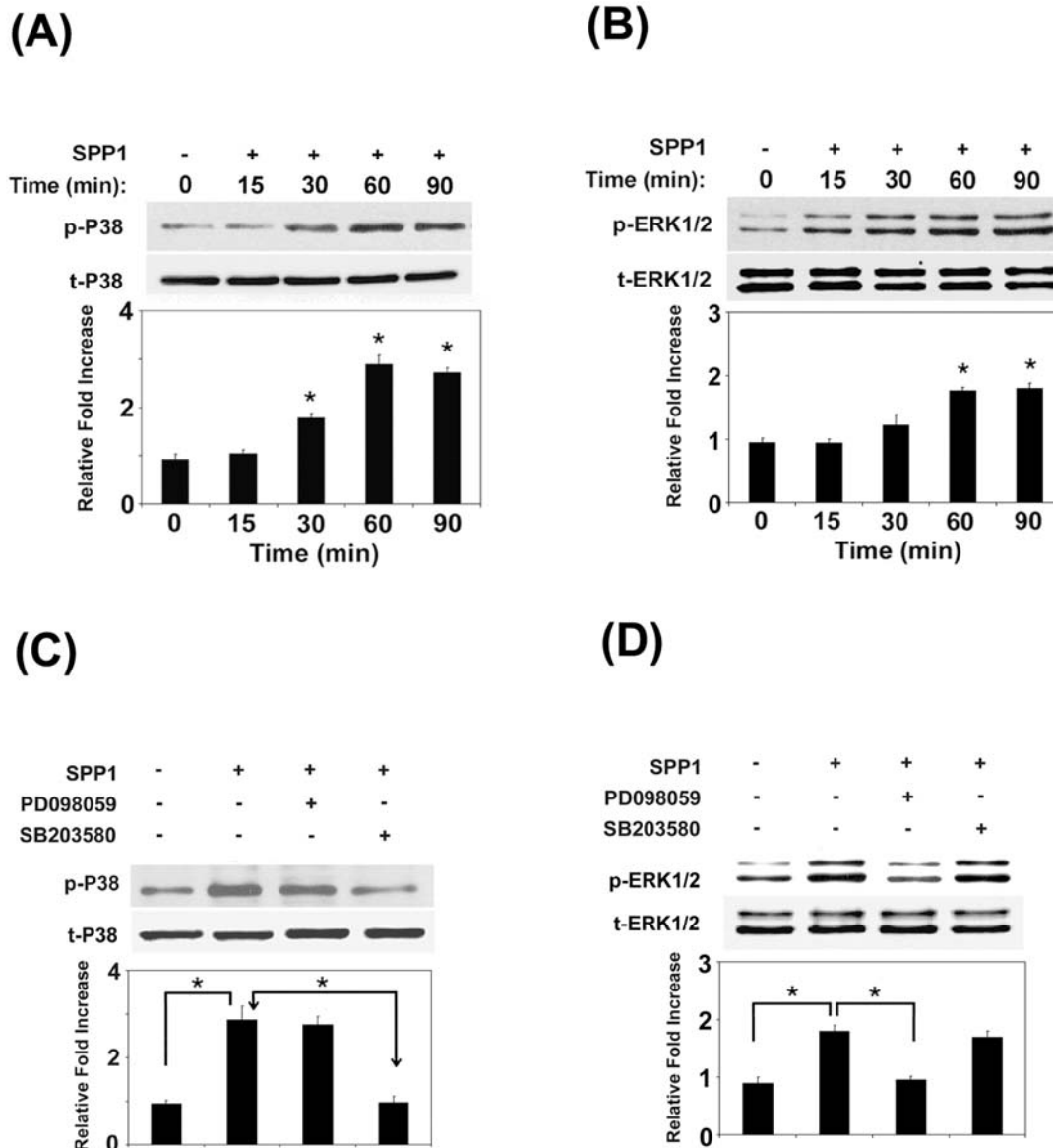


Fig. 4.3. SPP1 binds integrins to activate mTOR, P70S6K and Erk1/2 signal transduction in oTr1 cells. A, Detection of p38; B, Detection of Erk1/2; C and D, Detection of p-p38 and p-Erk1/2 in stimulated cells with SPP1 for 60 min after pretreatment with SB203580 and PD098059 for 30 min; tubulin A (TUBA) was detected as a total protein control; E, Detection of p-P70S6K following pharmacological inhibition of intracellular signaling with SB203580 at 10 and 30 μ M; F, Immunoprecipitation with P70S6K antibody and detection with p38 antibody; G, Detection of P70S6K following pharmacological inhibition with SB203580 at 5, 10 and 30 μ M and immunoprecipitation with p38 antibody; H, Detection of p-P70S6K and p38 following 30 or 60 min treatment with 100 ng/ml of wild-type recombinant rat SPP1 (rSPP1 (RGD)) or recombinant rat SPP1 with a mutated integrin binding sequence (rSPP1 (RAD)). Asterisks indicate statistically significant differences among treatments ($P < 0.05$).

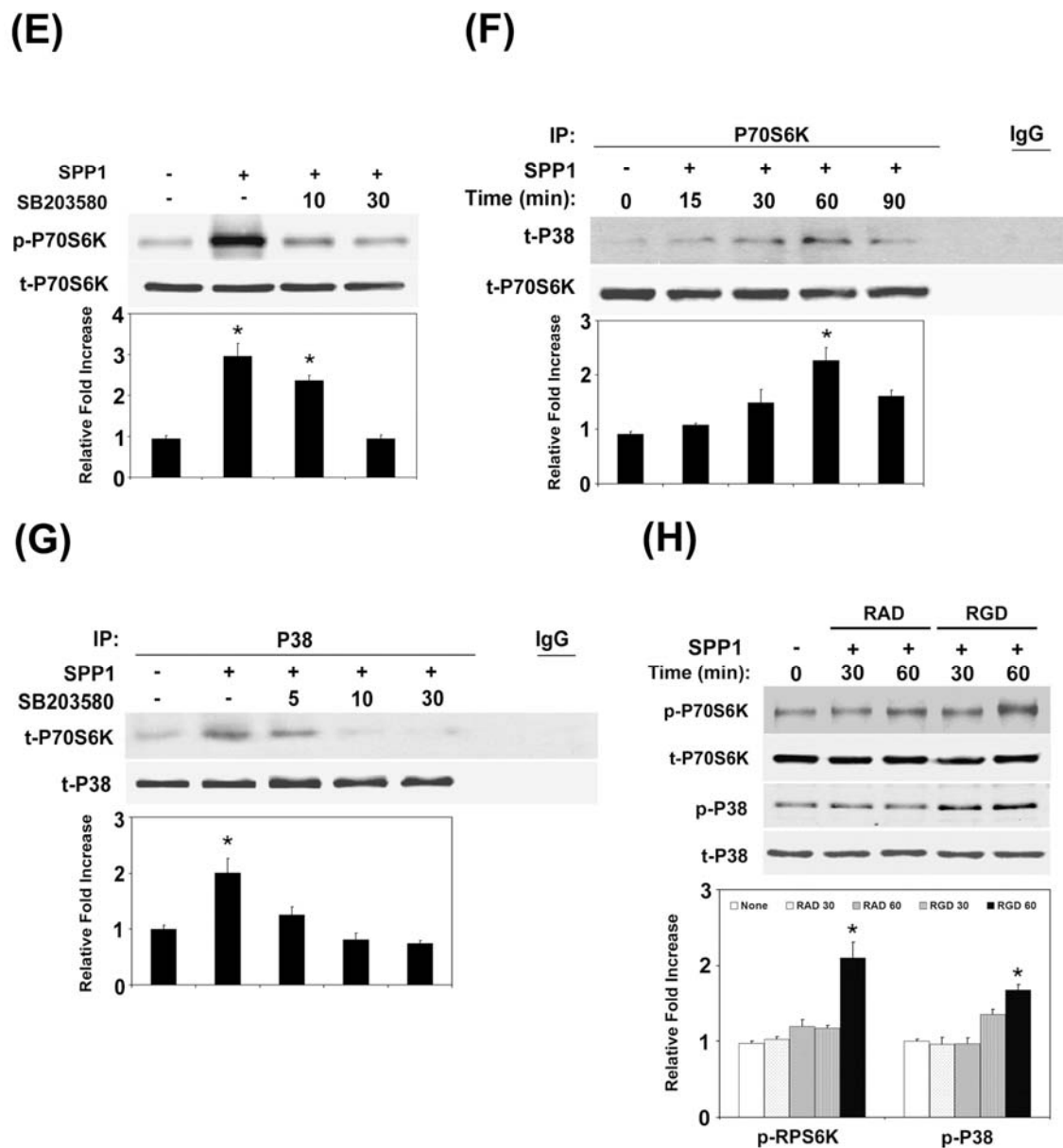


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and SB203580, a specific inhibitor of p38. Western blot analysis of whole oTr1 cell extracts with an antibody to p-P70S6K indicated that bSPP1 increased p-P70S6K by 2.7-fold ($P < 0.01$) and that this effect of bSPP1 was completely inhibited by SB203580 within 30 min ($P < 0.01$) (Fig. 4.3E). To demonstrate a direct interaction between p38 and P70S6K, extracts of bSPP1-treated oTr1 cells were immunoprecipitated with an antibody to P70S6K and analyzed by Western blotting using an antibody to p38. In response to bSPP1, p38 increased 1.8-fold ($P < 0.01$) by 60 min; levels of p38 decreased at 90 min compared to 60 min, but remained higher than basal levels (Fig. 4.3F). In a similar experiment, extracts of oTr1 cells treated with bSPP1 and SB203580 were immunoprecipitated with an antibody to p38 and analyzed by Western blotting using an antibody to P70S6K. bSPP1 induction of P70S6K was inhibited in a dose-dependent manner by SB203580 ($P < 0.01$) (Fig. 4.3G). These results indicate an interaction between P70S6K and p38 for crosstalk between the mTOR and MAPK signaling pathways.

SPP1 activates integrin-dependent phosphorylation of P70S6K and p38 in trophoctoderm cells

mTOR signaling has been linked to focal adhesions (266) and SPP1 binds to $\alpha\text{v}\beta 3$ and possibly $\alpha 5\beta 1$ integrins via its GRGDS integrin-binding amino acid sequence. Therefore, oTr1 cells were treated with either recombinant wild-type rat SPP1 (rSPP1(RGD)) or recombinant rat SPP1 containing a mutation of the RGD attachment sequence to RAD (rSPP1(RAD)). Western blot analyses of whole oTr1 cell extracts with antibodies against p-P70S6K and p-p38 proteins indicated that rSPP1(RGD) increased p-P70S6K by 2.2-fold ($P < 0.01$) and p-p38 by 1.7 fold ($P < 0.01$) (Fig. 4.3H). In contrast, rSPP1(RAD) did not increase either p-P70S6K or p-p38 within 60 min (Figure 3J). These results indicate that SPP1 binds integrins via its RGD sequence to activate P70S6K and p38 signaling pathways in oTr1 cells.

SPP1 stimulates adhesion and migration, but not proliferation of trophoctoderm cells

Because $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ integrin heterodimers on oTr1 cells bind SPP1 (Fig. 4.1), oTr1 cells were allowed to attach to various concentrations of SPP1 immobilized on 96-

well culture plates. As shown in Fig. 4.4A, bSPP1 and rSPP1(RGD) promoted greater attachment of oTr1 cells than human fibronectin (hFN) at all concentrations tested. In addition, rSPP1(RAD) did not promote oTr1 cell attachment, suggesting that the RGD integrin-binding amino acid sequence mediates attachment of oTr1 cells to SPP1. Integrin heterodimers require divalent cations for functional binding to ligands (279). Therefore, the oTr1 cell adhesion assay was performed in the presence or absence of physiological levels of divalent cations (2 mM Ca^{2+} and 1 mM Mg^{2+}) (280). Results indicated that oTr1 cell attachment to SPP1 and hFN ($P < 0.05$) was integrin-mediated (Fig. 4.4B). These results, coupled with the requirement for the RGD sequence in SPP1, indicate that adhesion of oTr1 cells to SPP1 occurs through an RGD-mediated integrin mechanism. To confirm that $\alpha\text{v}\beta 3$ and possibly $\alpha 5\beta 1$ integrins are required for SPP1-mediated oTr1 cell adhesion, cell attachment experiments were conducted by incubating oTr1 cells in the presence of function-blocking antibodies directed to the $\alpha 5$ integrin subunit, the $\alpha\text{v}\beta 3$ integrin heterodimer or $\alpha 2\beta 1$ integrin heterodimer (negative control) alone or in combination before being allowed to attach to plates coated with bSPP1. The $\alpha 2\beta 1$ integrin heterodimer is commonly expressed on epithelial cells, but does not bind SPP1. Cell adhesion was significantly inhibited when oTr1 cells were incubated with an antibody to the $\alpha\text{v}\beta 3$ integrin heterodimer, compared to the $\alpha 2\beta 1$ blocker ($P < 0.05$) (Fig. 4.4C). A slight reduction in adhesion occurred due to the $\alpha 5$ blocking antibody that was not significantly enhanced by addition of $\alpha 2\beta 1$ blocker. Combination of $\alpha 5$ and $\alpha\text{v}\beta 3$ antagonism reduced attachment slightly, but no additional reduction in attachment was observed when combining $\alpha\text{v}\beta 3$ and $\alpha 2\beta 1$ blocking antibodies. These results confirm that adhesion of oTr1 cells to SPP1 is mediated by the $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ integrin heterodimers.

To evaluate effects of SPP1 on oTr1 cell migration, assays were performed with various concentrations of bSPP1. As shown in Fig. 4.4D, bSPP1 stimulated a dose-dependent increase in migration of oTr1 cells with the greatest effect at 100 and 1,000 ng/ml ($P < 0.05$). Therefore, stimulation of migration of oTr1 cells by SPP1 may be essential for conceptus elongation.

The effects of SPP1 on oTr1 proliferation were assessed on cells seeded directly on polystyrene wells at 50% confluency. bSPP1 did not stimulate oTr1 proliferation as

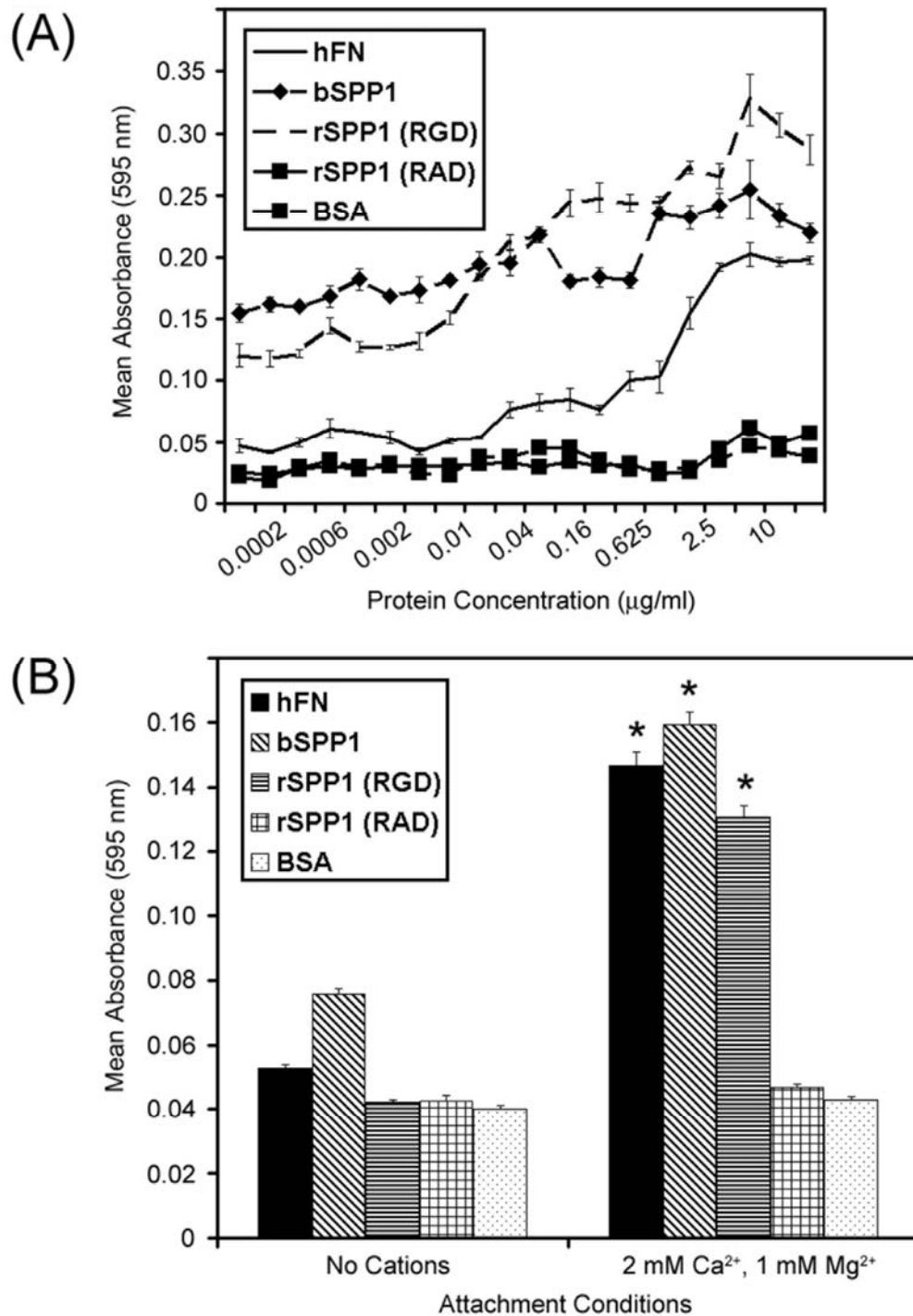


Fig. 4.4. SPP1 stimulates adhesion and migration, but not proliferation of oTr1 cells. A, Adhesion of oTr1 cells to increasing concentrations of immobilized SPP1; B, Adhesion of oTr1 cells to immobilized SPP1 in the presence or absence of divalent cations; C, Inhibition of adhesion using function-blocking integrin antibodies to specific integrins on oTr1 cells; D, Migration of oTr1 cells in response to treatment with soluble SPP1. E, Proliferation of oTr1 cells in response to treatment with soluble SPP1.

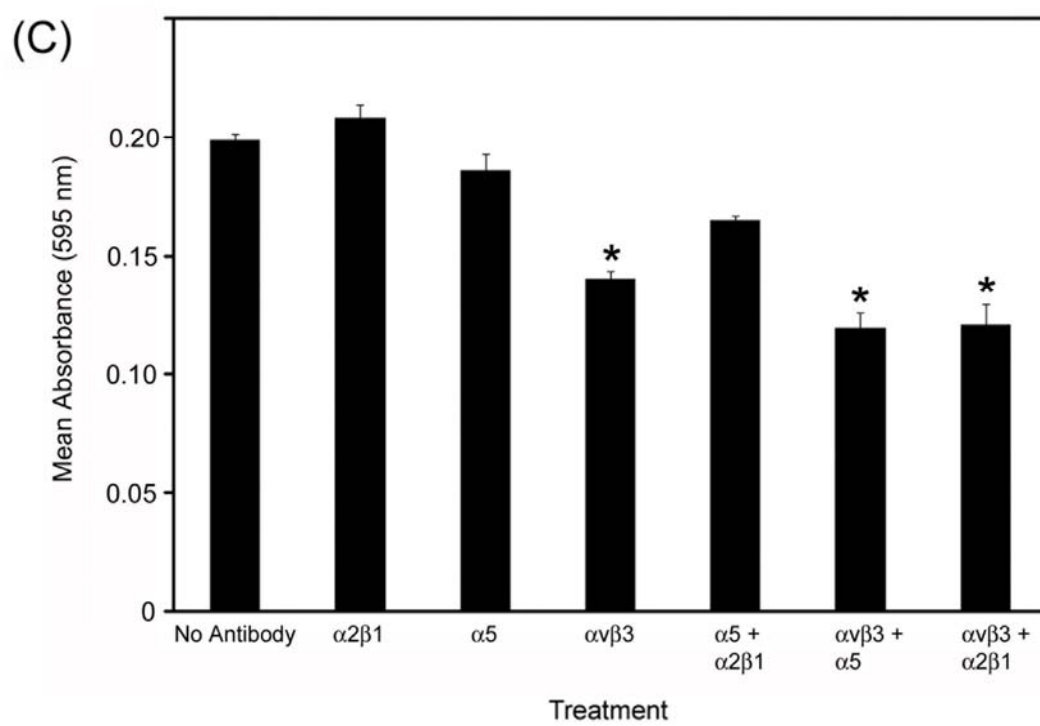


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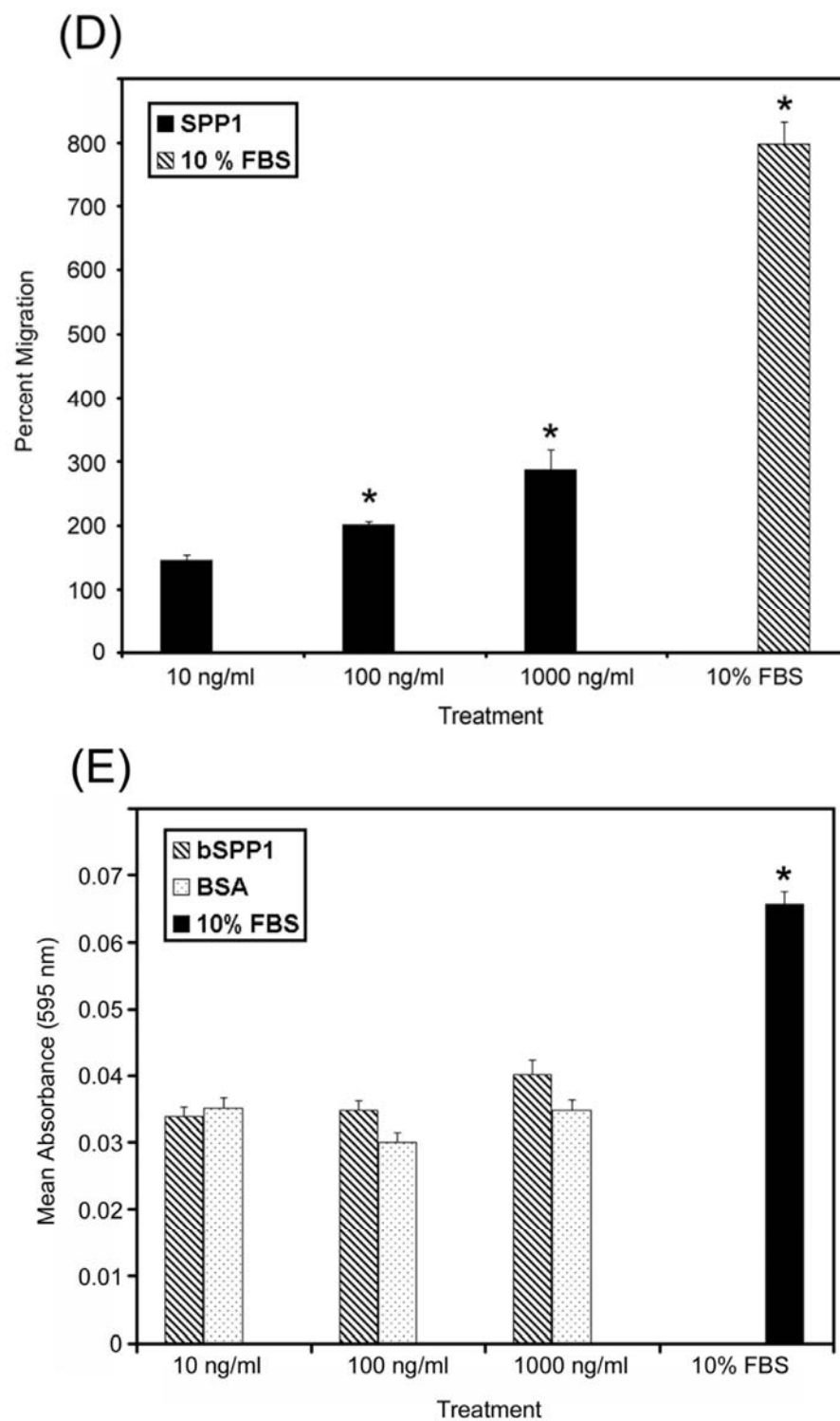


Fig. 4.4 continued.

compared to effects of fetal bovine serum (FBS) (positive control) or bovine serum albumin (BSA) (negative control) ($P < 0.05$) (Fig. 4.4E). Collectively, these experiments revealed that SPP1 stimulates adhesion and migration of oTr1 cells, events which are essential for conceptus elongation and implantation.

SPP1 stimulates activation of integrin receptors to form focal adhesions at the apical surface of trophoderm cells

Implantation in all mammalian species is coincident with the unique apical expression of integrins on conceptus Tr and uterine LE that is hypothesized to mediate attachment between these apposing layers of epithelial cells. Therefore, it was essential to determine whether SPP1 mediates focal adhesion formation at the physiologically relevant apical surface of oTr1 cells. Integrin aggregation and outside-in signaling was found to occur in response to rSPP1(RGD) immobilized on polystyrene beads placed at the apical surface of oTr1 cells plated on collagen Type I (which does not bind α_v or α_5 integrins) (Fig. 4.5). These apical focal adhesions are represented by immunofluorescence co-localization of the integrin α_v subunit with TLN and result from integrin activation in response to binding of SPP1 on the surface of the bead. TLN is an essential and early component in focal adhesion assembly and a key indicator of integrin activation (281). Basal focal adhesions served as an internal negative control for integrin α_v and positive control for TLN localization. No apical focal adhesions were induced by beads coated with rSPP1(RAD) containing a mutated integrin binding sequence, as evidenced by lack of integrin α_v and talin aggregation around the bead. These results demonstrate that SPP1 induces cytoskeletal rearrangement and focal adhesion formation necessary for attachment and migration of oTr1 cells.

Functional blockade of adhesion and migration of oTr1 cells with inhibitors of integrin binding, myosin II motor activity, and mTOR/PI3K/Erk1/2/p38 signaling

Previous results indicated that SPP1 binds the $\alpha_v\beta_3$ and possibly $\alpha_5\beta_1$ integrins to induce focal adhesion formation and activate multiple signaling pathways for adhesion and migration of oTr1 cells. Therefore, it was determined if oTr1 cell migration could be blocked by using: 1) a functional blocker of myosin II motor activity (blebbistatin); 2)

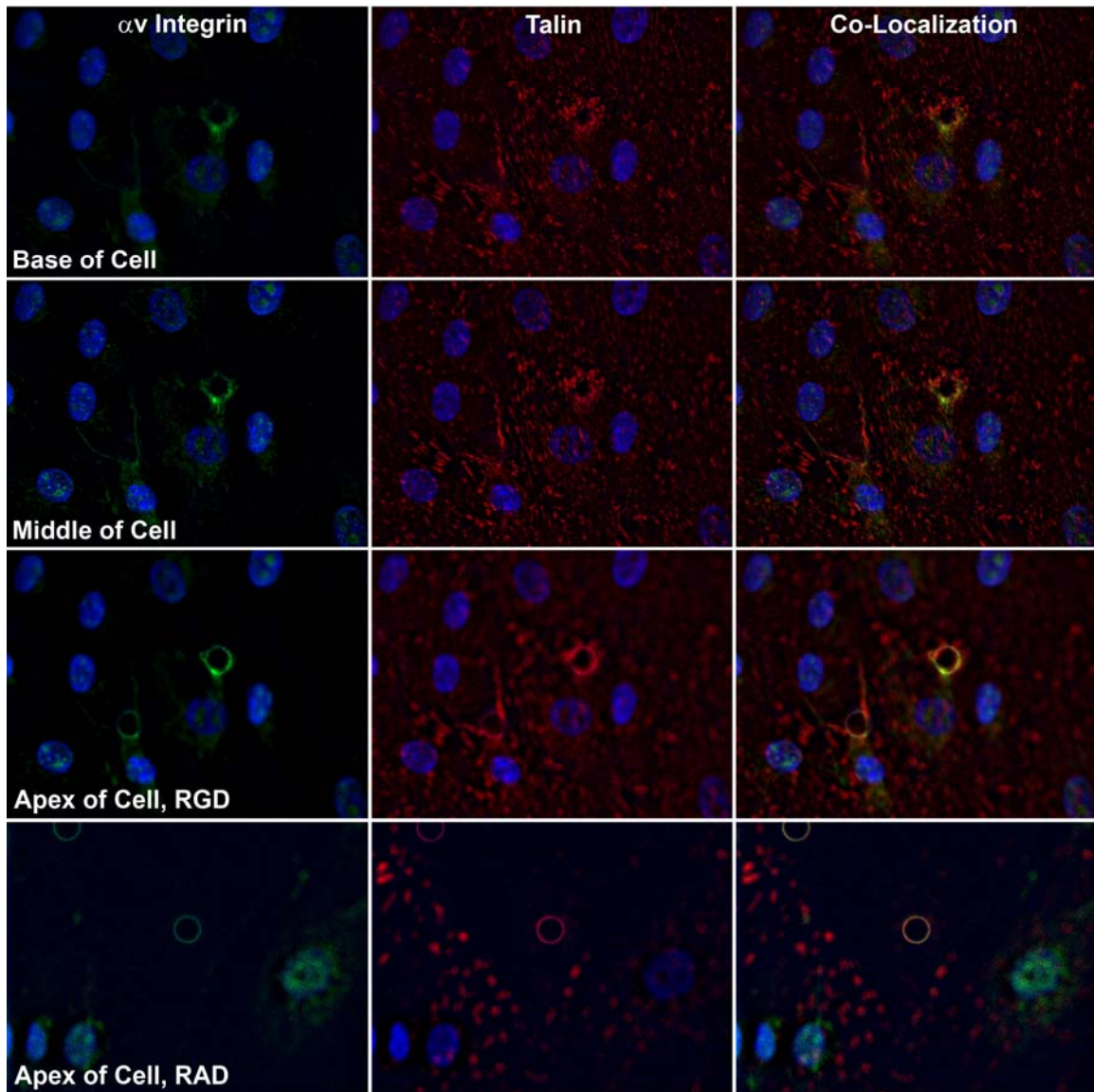


Fig. 4.5. SPP1 stimulates activation of integrin receptors to form focal adhesions at the apical surface of oTr1 cells. Immunofluorescence co-localization of integrin α_v subunit with TLN following incubation of beads coated with either rSPP1(RGD) (intact integrin binding sequence) or rSPP1(RAD) (mutated integrin binding sequence). Top panels show images taken at the base of the cell; middle panels show images taken from the middle of the cell where integrin aggregation and cytoskeletal rearrangement is occurring around the bead; bottom panels show the apex of the cell. Beads coated with rSPP1(RAD) did not induce focal adhesion as indicated by absence of integrin α_v and talin aggregation around the bead (bottom panels).

an inhibitor of mTOR signaling (rapamycin); 3) an inhibitor of p38 signaling (SB203580); 4) an inhibitor of Erk1/2 signaling (PD098059); and/or 5) an inhibitor of PI3K signaling (LY294002).

Blebbistatin inhibits the ability of myosin II to interact with actin, thus altering the contractile forces generated between the cytoskeleton and cell membrane necessary for successful cell migration. Addition of blebbistatin alone or in combination with rapamycin, SB203580, PD098059 or LY294002 ablated oTr1 cell migration ($P < 0.05$) (Fig. 4.6A-C). In addition, oTr1 cells incubated on immobilized bSPP1 and treated with increasing amounts of blebbistatin exhibited decreased cell spreading (Fig. 4.6D). These results indicate that SPP1-induced migration of oTr1 cells requires integrin activation and cytoskeletal force generation. Because inhibition of focal adhesion motor activity ablated migration, the individual contributions of the various signaling pathways listed previously were assessed using the specific inhibitors alone or in combination in the absence of blebbistatin. The addition of rapamycin, SB203580, PD098059 or LY294002 reduced ($P < 0.05$) oTr1 cell migration as compared to control oTr1 cells not treated with SPP1 (Fig. 4.6B), although there were no significant ($P > 0.05$) differences among effects of the individual inhibitors. However, specific combinations of inhibitors further decreased migration as compared to effects of individual inhibitors (Fig. 4.6C). Addition of SB203580 or PD098059 to rapamycin-treated oTr1 cells further decreased migration by 24% and 20%, respectively ($P < 0.05$). Addition of PD098059 to SB203580-treated cells further decreased migration by 13% ($P < 0.05$). The combination of SB203580 and PD098059 further decreased migration of oTr1 cells compared to effects of rapamycin alone (33%), SB203580 alone (14%) or PD098059 alone (19%) ($P < 0.05$). Thus, mTOR, p38, PI3K and Erk1/2 each contribute to migration of oTr1 cells and their collective effects converge to facilitate optimal migration of oTr1 cells. Collectively, results of function blocking studies demonstrated that SPP1-induced adhesion and optimal motility of oTr1 cells requires integrin activation to form focal adhesions and subsequent intracellular signaling through mTOR/Erk1/2/p38.

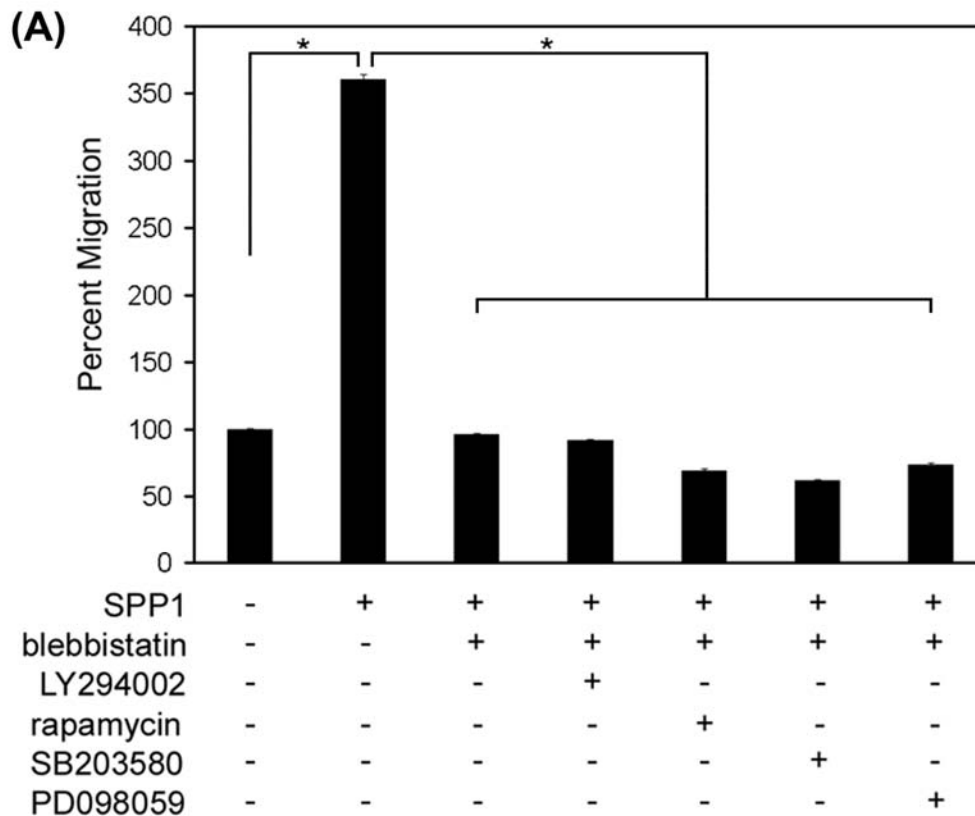


Fig. 4.6. Functional blockade of oTr1 cell migration with inhibitors to myosin II motor activity, and mTOR/PI3K/Erk1/2/p38 signaling. The oTr1 cells were incubated with soluble SPP1 in Transwell filter inserts and treated with pharmacological inhibitors of intracellular signaling: A, blebbistatin alone or in combination with LY294002, rapamycin, SB203580 or PD098059. The asterisks indicate that untreated control cells and cells treated with SPP1 plus blebbistatin or SPP1 plus blebbistatin in combination with an additional inhibitor exhibited statistically decreased migration when compared to cells treated with SPP1 alone ($P < 0.05$); B, Migration of oTr1 cells treated with LY294002, rapamycin, SB203580, PD098059 or blebbistatin were not different from untreated control cells. However, asterisks indicate that cells treated with SPP1 plus an individual inhibitor exhibited decreased migration when compared to cells treated with SPP1 alone ($P < 0.05$); C, LY294002, rapamycin, SB203580 or PD098059 in various combinations are indicated. The asterisks indicate that untreated control cells, and cells treated with SPP1 and a combination of two inhibitors exhibited decreased migration when compared to cells treated with SPP1 alone ($P < 0.05$); D, oTr1 cells cultured on immobilized SPP1 for 1h and treated with 0, 10, 25, or 50 μ M blebbistatin. Cells cultured in 10, 25 or 50 μ M blebbistatin exhibited impaired cell spreading after 1 h of treatment.

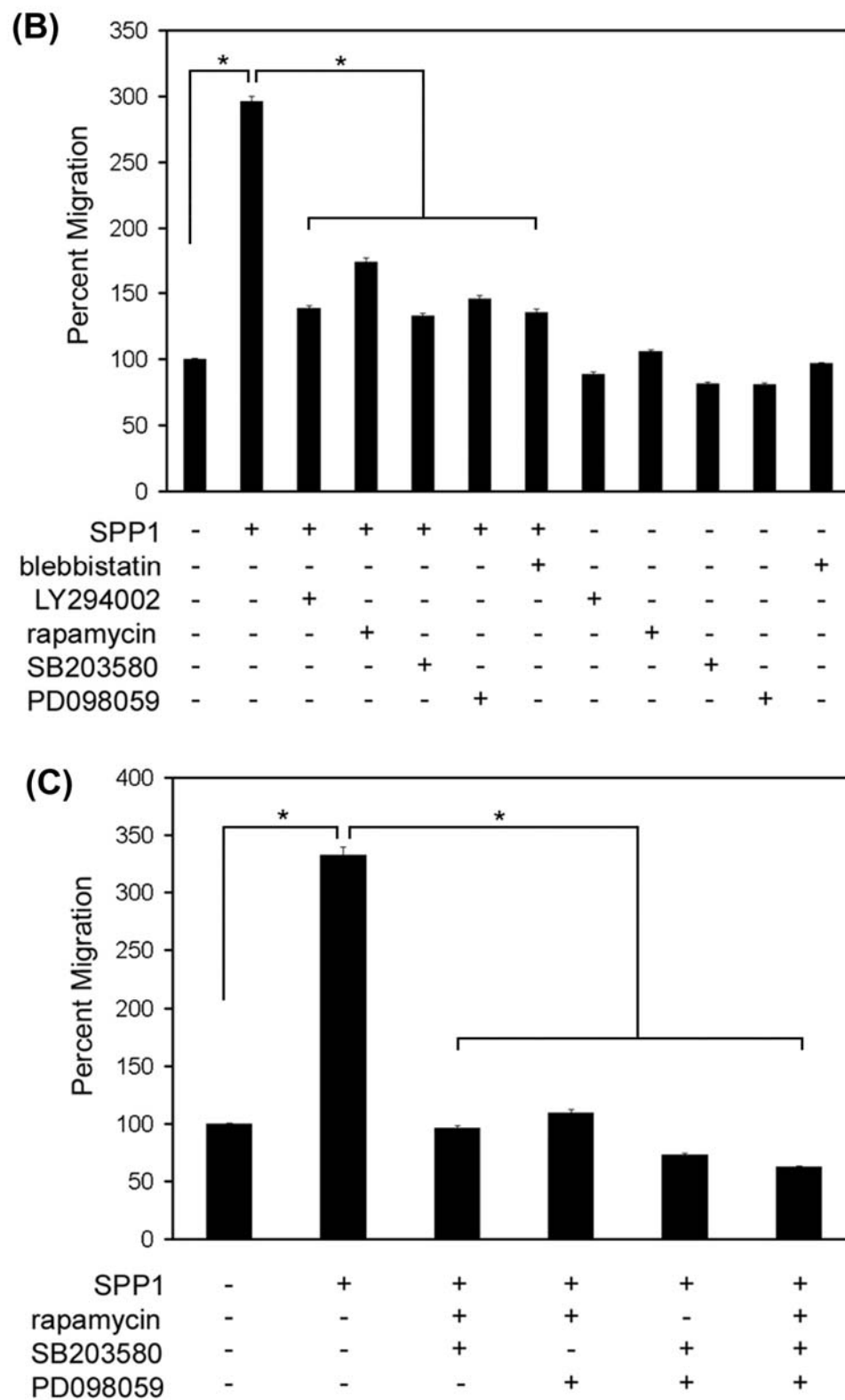


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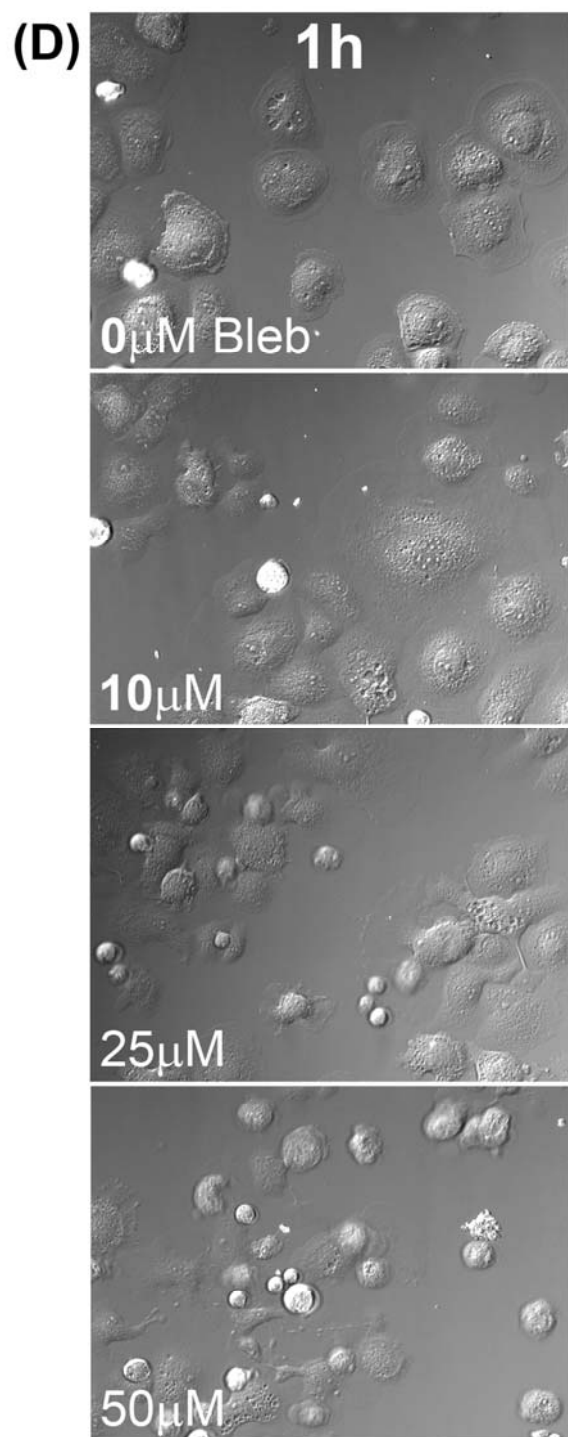


Fig. 4.6 continued.

Discussion

Growth and development of mammalian blastocysts during the peri-implantation period are prerequisites for implantation, a unique biological event requiring secretions from uterine glandular epithelia, which include SPP1 (6). Indeed, SPP1 in the uterine lumen has been linked to implantation in several species including sheep and humans (167). Multiple integrin receptors for SPP1 are present on Tr and LE of humans and domestic animals, some of which increase during the peri-implantation period (167, 182, 262). Results of the present study indicate that SPP1 binds the $\alpha v \beta 3$ and possibly $\alpha 5 \beta 1$ integrins to induce focal adhesion assembly as a prerequisite for adhesion and migration of Tr cells by activating: 1) P70S6K via crosstalk between mTOR and MAPK pathways; 2) mTOR, PI3K, Erk1/2 and p38 signaling to stimulate Tr cell migration; and 3) focal adhesion assembly and myosin II motor activity to induce migration of Tr cells. These cell signaling pathways, acting in concert, mediate adhesion, migration and cytoskeletal remodeling of Tr cells that is essential for expansion of blastocysts, elongation and attachment of Tr to uterine LE for implantation and placentation. As sheep are used extensively as the animal model for studies of pregnancy, the results presented here have translational significance to human pregnancy (282).

Implantation is a complex event whereby the apical surface of Tr cells of morphologically differentiating and growing conceptuses closely appose and attach to the apical surface of a relatively static uterine LE. The apical attachment of two epithelia is unique to implantation, and demands specialized modification of the architecture of Tr cells as they depolarize their apical domain to allow migration, hyperplasia and hypertrophy to occur coincidentally as they make contact across the surface of uterine LE. Factors affecting these early events in placental development are essential for the exchange of nutrients and gases required for survival and growth of trophoblast initially, and subsequently, fetal-placental tissues. Results of the present study demonstrate that SPP1 is a potent stimulator of adhesion and migration of Tr cells through binding to $\alpha v \beta 3$ and possibly $\alpha 5 \beta 1$ integrins leading to assembly of focal adhesions. Focal adhesions are physical and biochemical links between the cell and ECM that also serve as signaling centers that regulate multiple cellular signaling pathways. A focal adhesion is composed of an ECM ligand, integrin heterodimer and associated cytoskeletal proteins that work

together to transmit mechanical forces and regulatory signals from the ECM to the signaling machinery and cytoskeleton (283). A major finding of this study is the remarkably high level of adhesion of Tr cells to SPP1 (Fig. 4.4) compared to other proteins tested, including FN, requiring robust interactions between integrins on Tr cells and extracellular SPP1 to act as primary mediators for adhesion of Tr to uterine LE during conceptus development and implantation.

Results of the present study are novel in establishing that SPP1 binds integrins to induce cell signaling via PI3K-AKT1-mTOR-P70S6K-RPS6, Erk1/2 or p38 to affect adhesion and migration of Tr cells of mammalian conceptuses. Migration and adhesion are essential functions of Tr cells responsible for the overlapping events of elongation (livestock species), attachment to uterine LE (all mammals) and invasion into the uterine endometrium (rodents and primates). The PI3K-AKT1 cell signaling pathway has emerged as a critical component of cell signaling cascades induced by insulin and IGF in human endometrium and decidual cells (254), IGF2 in ovine conceptus Tr (268), leucine and arginine in mouse blastocysts (22), and glucose in human placenta (21). Our understanding of the role(s) of PI3K-AKT1 cell signaling during implantation is significantly advanced by results from the present study demonstrating that SPP1 induces activation of PI3K-AKT1 and phosphorylation of mTOR-P70S6K-RPS6, which likely complement cell signaling initiated by nutrients and mitogens (255). mTOR and PI3K-AKT1 pathways act in parallel to transduce growth factor signals and regulate common downstream targets such as P70S6K (242). In the present study, SPP1-induced phosphorylation of P70S6K was inhibited by LY294002 (PI3K-AKT1 inhibitor) and rapamycin (mTOR inhibitor). Therefore, we propose that the collective effects of SPP1, nutrients (i.e., leucine, arginine and glucose), and mitogens (i.e., insulin and IGF) are to activate the PI3K-AKT1 and mTOR-P70S6K-RPS6 cell signaling pathways that stimulate cell proliferation, differentiation and gene expression essential for survival, growth and differentiation of Tr cells during the peri-implantation period (see Fig. 4.7A and B).

Results of the present study further demonstrate that SPP1 activates Erk1/2 and p38 in oTr1 cells. These members of the MAPK family are highly conserved in organisms from yeast to humans (256). Among the well-characterized subfamilies of

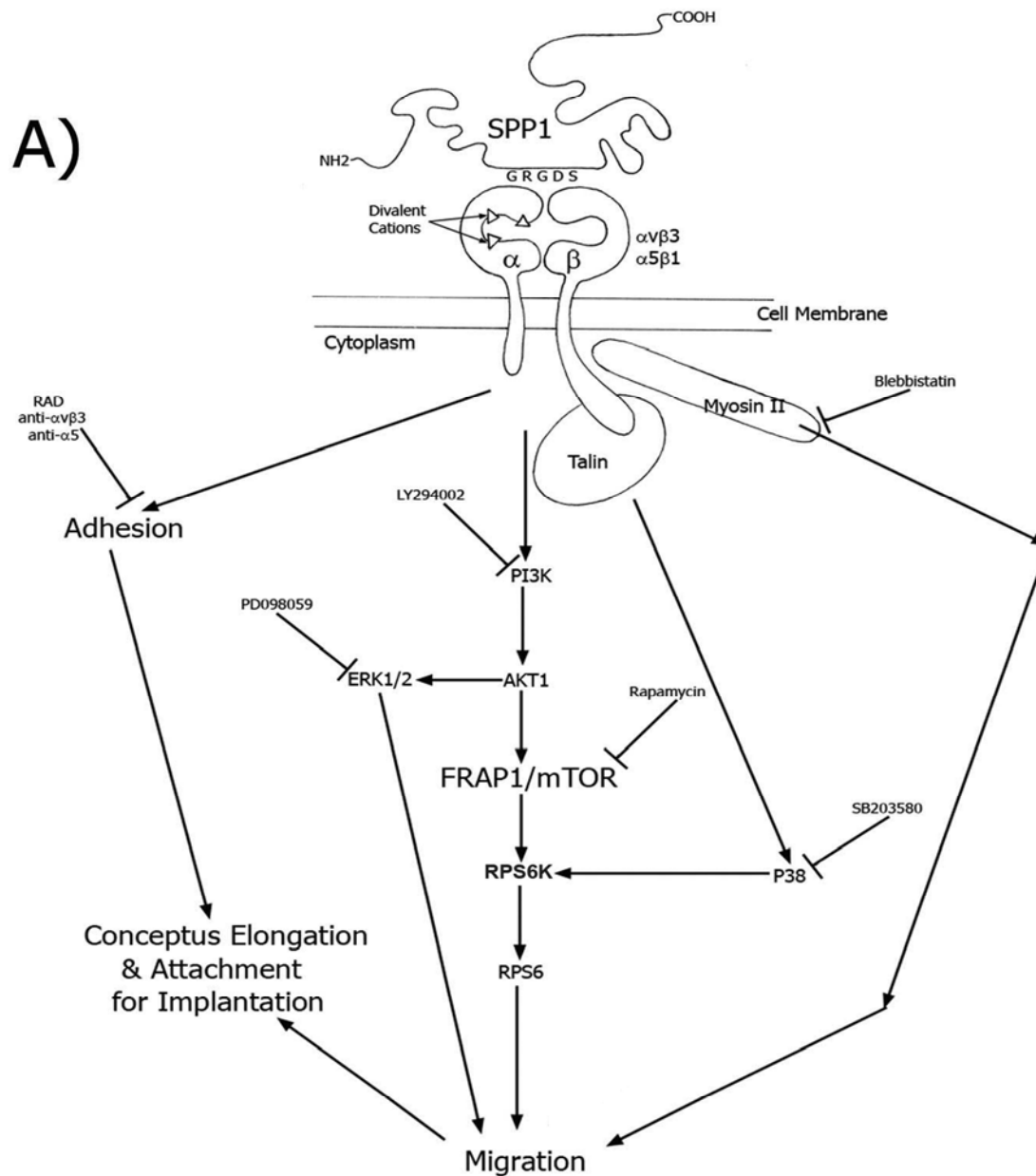


Fig. 4.7. SPP1 induction of intracellular signaling pathways in oTr1 cells: Regulation of essential physiological events for early pregnancy. A, Model illustrating that SPP1 acts via its RGD sequence to bind $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrin heterodimers to induce focal adhesions, as well as mTOR, p38 and Erk1/2 signaling to affect cell adhesion and migration. These events are essential for conceptus elongation and adhesion during the peri-implantation period of early pregnancy. B, A working model summarizing pathways where L-arginine, SPP1 and IGF2 may activate mTOR cell signaling pathways involving AKT1, TSC1/2 and mTORC1 (cell proliferation and gene expression) or mTORC2 (cell migration, cell survival and cytoskeletal organization) to affect Tr of conceptuses transitioning from spherical to tubular and filamentous forms that can signal pregnancy recognition, and undergo implantation and placentation.

B)

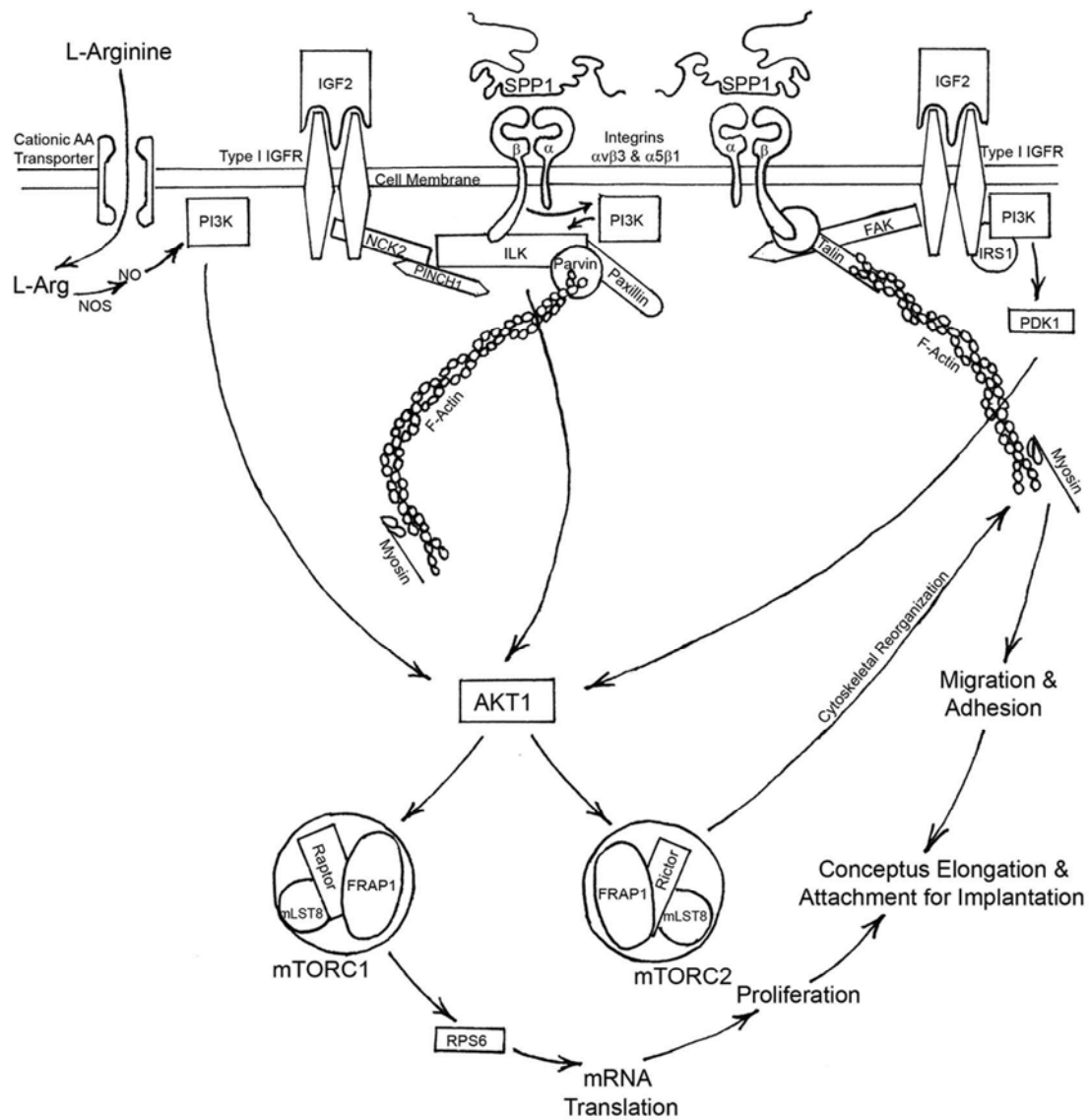


Fig. 4.7 continued.

MAPKs, Erk1/2 and p38 pathways play important roles in differentiation processes, including embryonic and placental development (232, 259, 284). Results of the present study provide the first evidence that SPP1 induces rapid phosphorylation of both Erk1/2 and p38, as well as crosstalk between P70S6K and p38 in oTr1 cells. This crosstalk has only been reported for osteoblasts (285). Therefore, SPP1 influences ovine conceptus development by simultaneously stimulating the mTOR-P70S6K-RPS6 and MAPK pathways during the peri-implantation period of pregnancy.

Another exciting finding of the present study is that SPP1 induces PI3K-AKT1-mTOR-P70S6K-RPS6 or Erk1/2 or p38 cell signaling to affect migration of Tr cells via a mechanism requiring formation of focal adhesions. The ability of blebbistatin to inhibit SPP1-induced Tr cell migration through PI3K-AKT1-mTOR-P70S6K-RPS6 or Erk1/2 or p38 cell signaling strongly suggests that SPP1 changes tensional integrity (tensegrity) of Tr cells as they elongate and attach to uterine LE during implantation. Cells use tensegrity architecture to control shape and structure through changes in stability of cytoskeletal elements that include microfilaments, intermediate filaments and microtubules (286). Integrins and associated proteins are regarded as tensegrity structures because molecular connections between ECM, integrins, cytoskeletal filaments and nuclear scaffolds provide a discrete path for mechanotransduction through cells as well as a mechanism for producing integrated changes in cellular and nuclear structure (286). The resulting cytoskeletal complex or focal adhesion physically links integrins to the ends of contractile microfilament bundles (“stress fibers”) to form a molecular bridge between ECM and cytoskeleton. In this investigation blebbistatin was used as a highly specific small molecule inhibitor with high affinity and selectivity toward myosin II which functions by blocking myosin II in an actin-detached state. Blebbistatin is not known to affect other components or mechanisms of the complex cell migration process. Because myosin II is believed to be one of the main components producing the force required in the process of cell migration, the specificity of this inhibitor indicates the involvement of the actomyosin-based contractility of oTr1 cell migration. Indeed, stimulation of integrin aggregation and recruitment of the cytoskeletal protein TLN around an SPP1-coated bead indicates that SPP1 can facilitate the reshaping of the cytoskeleton of Tr cells necessary for cell migration.

Based on results from the present studies, a model had been proposee (Fig. 4.5A-B) in which: 1) SPP1 serves as a key adhesive ECM protein that mediates Tr cell attachment, focal adhesion formation and reorganization of the cytoskeleton necessary for cell migration and attachment to uterine LE; 2) IGF2 and SPP1 activate PI3K and MAPK signaling pathways to affect Tr migration necessary for cellular and tissue reorganization during conceptus elongation (268); and 3) arginine induces proliferation of Tr cells required for conceptus development (22, 96); Chapter V of this dissertation). Collectively, SPP1, IGF2 and arginine activate mTOR signaling pathways mediated by AKT1, TSC1/2 and mTORC1 (cell proliferation and mRNA translation), as well as mTORC2 (cell migration, cell survival and cytoskeletal organization) in Tr cells to allow conceptuses to make the critical transition from spherical to tubular and filamentous forms that is a prerequisite for signaling pregnancy recognition, implantation and placentation required for successful pregnancy outcomes in all mammals.

CHAPTER V

EFFECTS OF ARGININE, LEUCINE, GLUTAMINE AND GLUCOSE ON TROPHECTODERM CELL SIGNALING, PROLIFERATION AND MIGRATION *IN VITRO*

Introduction

During the peri-implantation period, blastocysts undergo a morphological transition from spherical to tubular and then elongated filamentous forms that develop into conceptuses (embryo/fetus and its associated extra-embryonic membranes) (4, 41). These elongation steps in conceptus development are supported by secretions from endometrial epithelia referred to as histotroph that include growth factors, hormones, cytokines, ions, lipids, glucose and amino acids that derive primarily in response to direct or indirect effects of progesterone (66). In sheep, studies of the uterine gland knockout ewe (UGKO) established that secretions of the endometrial glandular epithelium are essential for conceptus survival, elongation of trophectoderm and initiation of pregnancy recognition signaling (5, 6, 63, 68).

Amino acids serve as essential precursors for the synthesis of proteins and many biologically active substances including polyamines, nitric oxide, peptides, neurotransmitters, nucleotides and creatine (287-289). Amino acids also function as regulators of hormone secretion (290, 291), as major energy sources for fetal-placental growth (201, 202, 215, 292), and as cell signaling molecules (119, 205, 287). In particular, nitric oxide, synthesized from L-Arg, is metabolically versatile and has potential physiological roles in regulating placental angiogenesis (293) and uterine blood flow during gestation (294, 295). Polyamines also derive from Arg. In this pathway Arg is metabolized to ornithine and ornithine is converted to polyamines by actions of ornithine decarboxylase. The polyamines are essential for placental development and mammalian embryogenesis (296). Further, Arg and/or Leu have pivotal roles in outgrowth of trophectoderm required for blastocyst motility and implantation in mice (22, 96). Glucose and Gln are major energy sources for conceptuses during development as they regulate trophoblast cell proliferation and function through the glutamine-fructose-

6-phosphate-amidotransferase (GFAT)-mediated target of rapamycin (MTOR, formerly FRAP1) signaling pathway (21).

In support of a crucial role for amino acids and glucose in embryogenesis and conceptus growth and development, we reported that: 1) total recoverable amounts of Arg, Leu, Gln and glucose increase in the uterine lumen by 13-, 10-, 15- and 7-fold in pregnant, but not cyclic ewes between Day 10 and Day 15 of after onset of estrus (95); 2) transporters for cationic, neutral and acidic amino acids are expressed or increase in uterine epithelia and conceptuses (212, 297); and 3) the abundance of both facilitative (SLC2A1) and a sodium-dependent (SLC5A1) glucose transporters increase in uterine epithelia and conceptus trophoctoderm (SLC2A3) during the peri-implantation period (94, 298). A sufficient supply of nutrients to the conceptus may be particularly important for domestic livestock species including ruminants and pigs, which have synepitheliochorial and epitheliochorial placentae, respectively, and for conceptuses that undergo rapid elongation during a protracted peri-implantation period (23). Despite the requirement for histotroph, little is known about contributions of individual nutrients on cell signaling pathways in trophoctoderm cells and how nutrients stimulate conceptus development during pregnancy, particularly in sheep and other ruminants. Therefore, the purpose of the present study was to determine the molecular mechanisms by which Arg, Leu, Gln and glucose activate FRAP1-RPS6K-RPS6 in ovine trophoblast cells and their respective biological effects on proliferation and migration of those cells.

Materials and Methods

Cell culture

Mononuclear ovine trophoctoderm (oTr) cells from Day 15 conceptuses were isolated and cultured as described previously (268). One cell line, referred to as oTr, was cultured in DMEM-F12 that included 10% FBS, 50U penicillin, 50 µg streptomycin, 0.1 mM each for non-essential amino acids, 1 mM sodium pyruvate, 2 mM Gln, and 0.7 µM insulin. When the density of cells in dishes reached about 80% confluence, they were passaged at a ratio of 1:3 and frozen stocks of cells were prepared at each passage. For experiments, monolayer cultures of oTr cells (between passages 9 and 13) were grown in culture medium to 80% confluence on 100 mm tissue culture plates. Cells were serum

starved for 24 h in customized medium that was made based on the levels of each of the select nutrients in fetal allantoic fluid (90). For experiments, the medium contained approximately one-tenth the amount of each amino acid and glucose compared to culture medium, and was deprived of either Arg, Leu, Gln or glucose for 6 h and then treated with Arg, Leu, Gln or glucose in a dose- and time-dependent manner. This design was replicated in three independent experiments.

Immunofluorescence analyses

The effects of amino acids and glucose on phosphorylation of RPS6K and RPS6 were determined by immunofluorescence microscopy as previously described (234, 264). Briefly, oTr cells were grown in Lab-Tek four-well chamber slides (Nalge Nunc International, Rochester, NY) and treated with Arg (0.2 mM), Leu (0.2 mM), Gln (0.5 mM) or glucose (4 mM) for either 30 or 60 min. At the indicated times, cells were fixed for 10 min in paraformaldehyde, rinsed, blocked in 5% normal goat serum, and incubated in primary antibody overnight at 4 C. Rabbit IgG was substituted for the primary antibody as a negative control. Cells probed with rabbit anti-human polyclonal phospho-RPS6K IgG (Thr421/Ser424) (Catalog number 9204) at a 1:200 dilution or rabbit anti-human polyclonal phospho-RPS6 IgG (Catalog number 2215) at a 1:200 dilution (both antibodies were from Cell Signaling Technology, Danvers, MA and used for western blotting) were incubated with goat anti-rabbit IgG Alexa 488 (Chemicon) at a 1:200 dilution for 1 h at room temperature. Cells were washed in PBS/Tween before incubation overnight in mouse anti-RPS6 (Catalog number 2317, Cell Signaling Technology, Danvers, MA) at a 1: 200 dilution at 4°C. Cells were then washed, incubated in goat anti-mouse IgG Alexa 594 at a 1:200 dilution and overlaid with Prolong Gold Antifade with DAPI. Slides were stored at 4°C in the dark before microscopic analyses. Fluorescence images of cells after treatment were captured using Axioplan 2 microscope with an AxioCam HR digital camera and Axiovision 4 software. For each primary antibody, images were captured with identical microscope and detector settings to facilitate comparisons of spatial distribution and fluorescence intensity differences between treatments.

Western blot analyses

Whole cell extracts and immunoblot assays were prepared and performed as described previously (234, 268) . To harvest total cellular protein for western blot analyses, oTr cells were rinsed with cold PBS and lysed by incubation in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4 , 0.2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 30 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 1 $\mu\text{g}/\text{ml}$ pepstatin) for 30 min at 4°C. Cell lysates were passed through a 26-gauge needle and clarified by centrifugation (16,000 x g, 15 min, 4°C). The protein content was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured, separated using SDS-PAGE, transferred to nitrocellulose and western blotting performed as described previously (268) using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and X-OMAT AR X-ray film (Kodak, Rochester, NY) according to manufacturer's recommendations. All antibodies used in these experiments were purchased from Cell Signaling Technology (Danvers, MA). Immunoreactive proteins were detected by using rabbit anti-mouse polyclonal phospho-AKT1 IgG (Ser473/Thr308) (Catalog numbers 9271 and 9275) at a 1:1,000 dilution and 10% SDS-PAGE gel, rabbit anti-human polyclonal phospho-RPS6K IgG (Thr421/Ser424) (Catalog number 9204) at 1:1,000 dilution and 10% SDS-PAGE gel, and rabbit anti-human polyclonal phospho-RPS6 IgG (Catalog number 2215) at 1:1,000 dilution and 12% SDS-PAGE gel. As a loading control, western blotting with mouse anti-alpha tubulin (TUBA) IgG (Catalog number; T5168; Sigma, St. Louis, MO) was performed. Multiple exposures of each western blot were conducted to ensure linearity of chemiluminescent signals. Western blots were quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA).

Proliferation assay

The oTr cells were subcultured in 24-well plates (50% confluent) in growth medium until the monolayer reached up to 80% confluency and then switched to serum- and insulin-free customized medium. After starvation for 24 h, cells were deprived of

amino acids or glucose for 6 h and treatments were then added to each well (n=3 wells per treatment) that included combinations of either: (a) serum- and insulin-free customized medium, a negative control; (b) Arg, Leu, Gln or glucose at the indicated doses with 5% serum or (c) 10 % serum containing medium as a positive control. The media were changed every 2 days and treated cells were maintained for 4 days. Cell numbers were determined as described previously (236, 299). Briefly, DMEM was removed from cells by vacuum aspiration, and cells were fixed in 50% ethanol for 30 min, followed by vacuum aspiration of the fixative. Fixed cells were stained with a Janus Green B in PBS (0.2% w/v) for 3 min at room temperature. The stain was immediately removed using a vacuum aspirator, and the whole plate was sequentially dipped into water and destained by gentle shaking. The remaining water was removed by shaking, stained cells were immediately lysed in 0.5N HCl, and absorbance readings were taken at 595 nm using a microplate reader. As described previously (299), cell numbers were calculated from absorbance readings using the following formula: cell number = (absorbance – 0.00462)/0.00006926. The entire experiment was independently repeated three times with different batches of oTr cells between passages 7 and 10.

Migration assay

Migration assays were conducted with oTr cells as described previously (268, 300). Briefly, oTr cells (50,000 cells per 100 µl serum and insulin-free DMEM) were seeded on 8 µm pore Transwell inserts (Corning Costar #3422, Corning, NY). Treatments that were identical to those previously used for western blotting and proliferation assays were then added to each well (n=3 wells per treatment). After 12 h, cells on the upper side of the inserts were removed with a cotton swab. For evaluation of cells that migrated onto the lower surface, inserts were fixed in 50% ethanol for 5 min. The transwell membranes were then removed, placed on a glass slide with the side containing cells facing up, overlaid with Prolong antifade mounting reagent with DAPI, and overlaid with a cover slip (Invitrogen-Molecular Probes, Eugene, OR). The migrated cells were systematically counted using a Zeiss Axioplan 2 fluorescence microscope with Axiocam HR digital camera and Axiovision 4.3 software (Carl Zeiss Microimaging,

Thornwood, NY). The entire experiment was repeated at least three times with different batches of oTr cells between passages 7 and 10.

Statistical analyses

All quantitative data were subjected to least squares analyses of variance (301) using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Western blot data were corrected for differences in sample loading using the TUBA data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P-value ≤ 0.05 was considered significant. Data are presented as least-square means (LSM) with standard errors (SE).

Results

Dose dependent phosphorylation of RPS6K by arginine, leucine and glucose, but not glutamine

The oTr cells deprived of amino acids or glucose for 6 h after serum starvation were treated with either Arg, Leu, Gln or glucose at the indicated doses (Fig. 5.1). Cells treated with Arg or Leu increased phosphorylation of RPS6K in a dose dependent manner over basal levels. Glucose, the primary metabolic fuel for conceptuses, also activated RPS6K, while Gln was ineffective. Basal levels of RPS6K at time 0 were similar in all groups; however, the exposure time was relatively short for Arg, Leu and glucose treated cells because the signal was strong enough to show the effect on phosphorylation of RPS6K. In subsequent experiments, oTr cells were treated with either Arg (0.2 mM), Leu (0.2 mM), Gln (0.5 mM), or glucose (4 mM).

Arginine, leucine and glucose, but not glutamine activate AKT1-mTOR-RPS6K-RPS6 signal transduction in ovine trophoblast cells

Western blot analyses of whole oTr cell extracts using antibodies to phosphorylated target proteins found that Arg increased ($P < 0.01$) pAKT1, pRPS6K and pRPS6 by 2.8-, 4.2-fold and 12-fold over basal levels, respectively, within 15 min, and this activation was maintained to 60 min (Fig. 5.2A). Leu stimulated a rapid 2.5-fold ($P <$

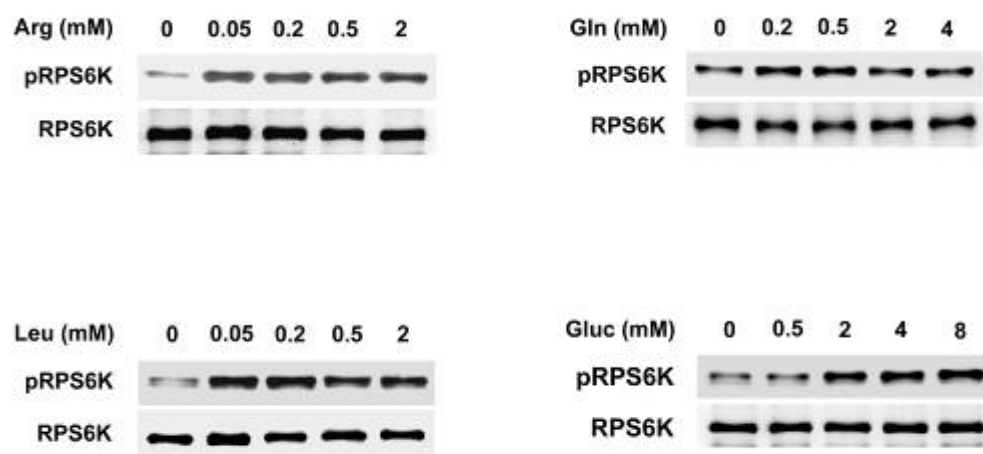


Fig. 5.1. Analysis of the phosphorylation of RPS6K in response to arginine (Arg), leucine (Leu), glutamine (Gln) and glucose (Gluc) in oTr cells. Monolayers of 80% confluent oTr cells were serum-starved for 24 h, deprived of amino acids or glucose for 6 h and then treated with one of the nutrients at the doses indicated. Blots were imaged to calculate the normalized values presented as levels of phosphorylated protein relative to total protein. In subsequent experiment, 0.2 mM Arg, 0.2 mM Leu, 0.5 Gln and 4 mM glucose was used.

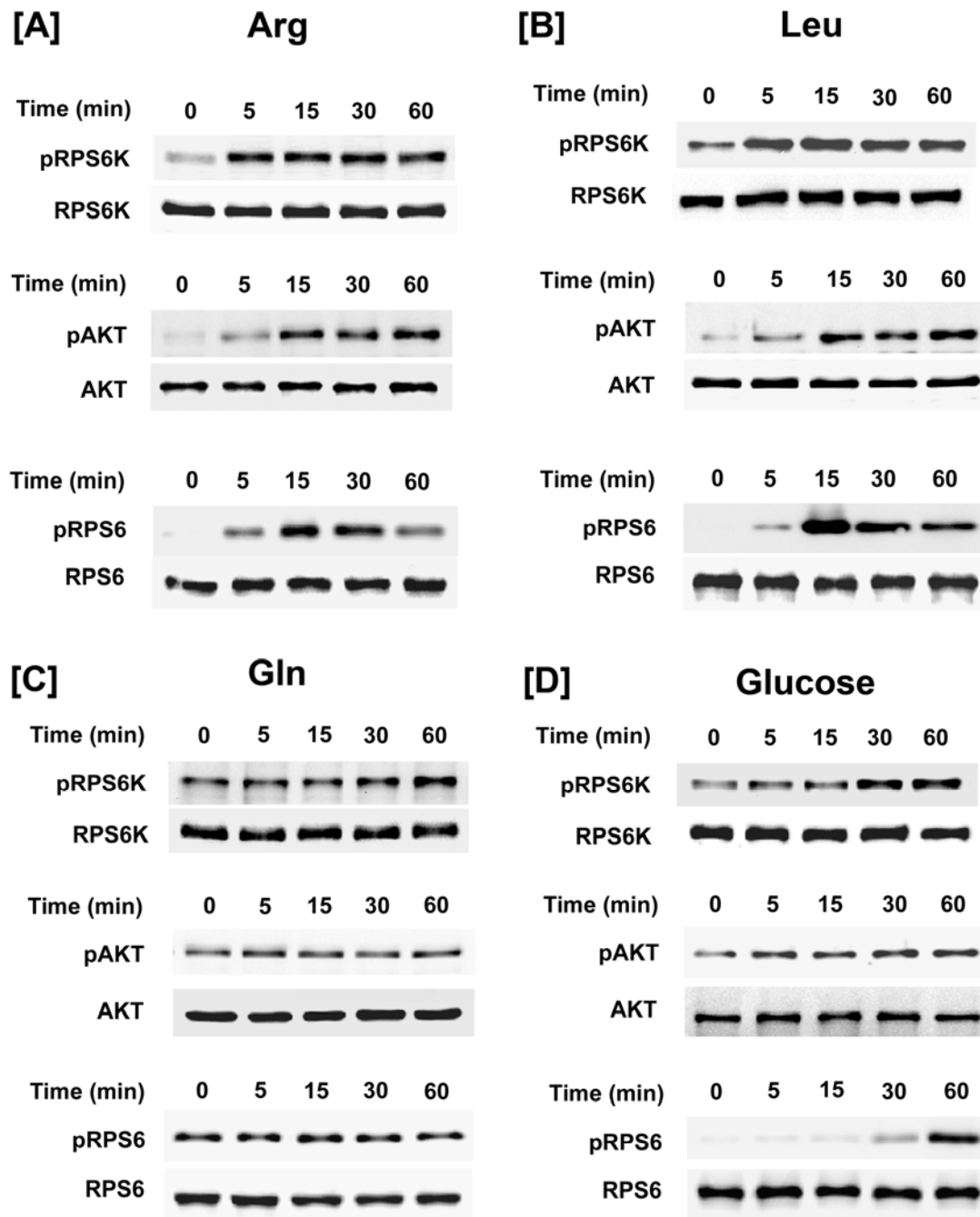


Fig. 5.2. Analysis of time dependent phosphorylation of AKT1, RPS6K and RPS6 in response to arginine (Arg), leucine (Leu), glutamine (Gln) and glucose (Gluc) in oTr cells at 0, 5, 15, 30 and 60 min after treatment, respectively in Panels A, B, C and D. Monolayers of 80% confluent oTr cells were serum-starved for 24 h and then deprived of either Arg, Leu, Gln or Gluc for 6 h and then treated with physiological concentrations of either Arg (0.2 mM), Leu (0.2 mM), Gln (0.5 mM), or glucose (4 mM). Blots were imaged to calculate the normalized values as levels phosphorylated protein relative to total protein.

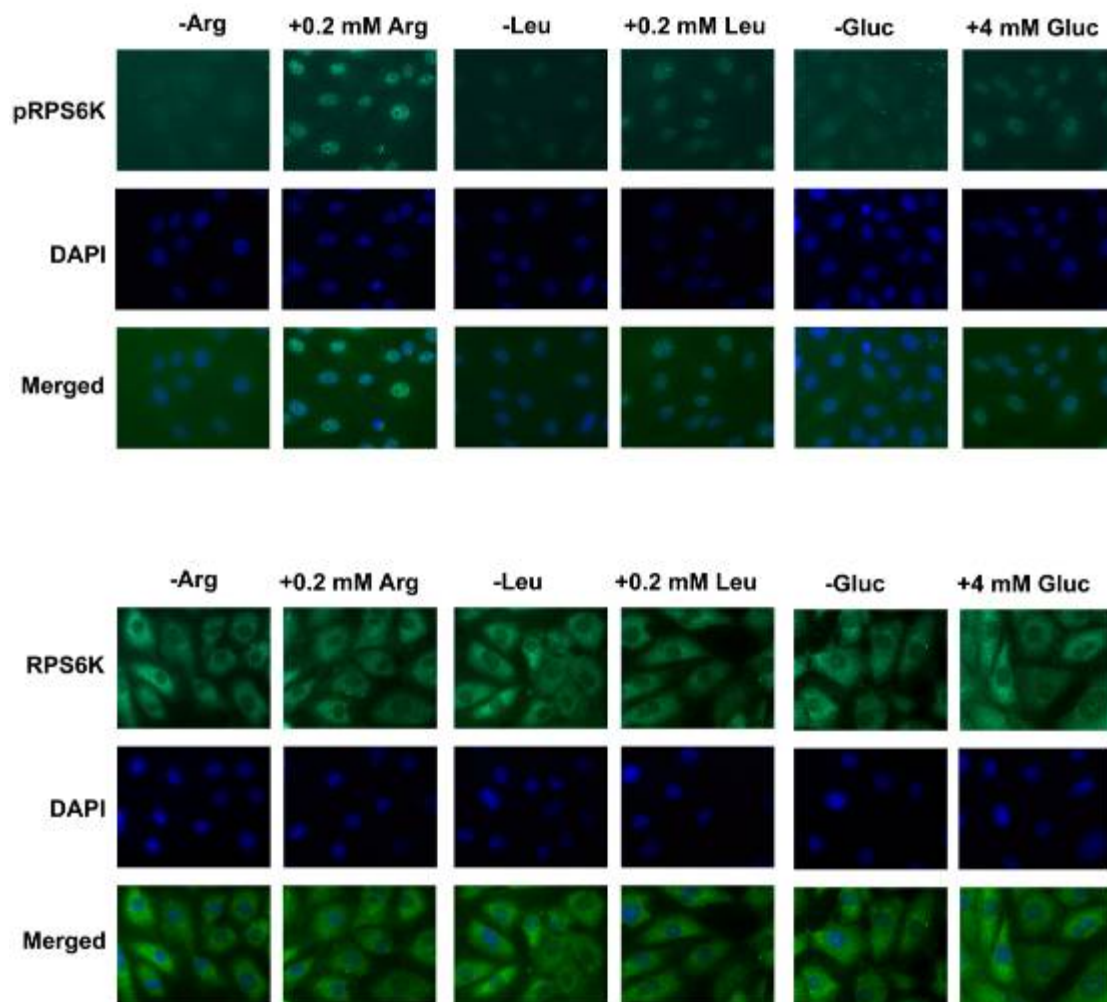


Fig. 5.3. Immunocytochemical localization of phosphorylated RPS6K protein in oTr cells. Immunoreactive pRPS6K protein was localized to the nucleus and/or cytoplasm of oTr cells using rabbit anti-human phospho-RPS6K (Thr421/Ser424) polyclonal antibody. The results indicate that only Arg-treated oTr cells exhibited translocation of pRPS6K protein to the nucleus whereas total RPS6K was primarily localized in cytoplasm.

0.01) increase in pAKT1, as well as increases in pRPS6K and pRPS6 by 4.7-fold ($P < 0.001$) and 15-fold ($P < 0.001$) within 15 min that were sustained through 60 min (Fig. 5.2B). Glucose also stimulated pAKT, pRPS6K and pRPS6 protein abundance by 1.8- ($P < 0.01$), 2.3- ($P < 0.005$) and 7-fold ($P < 0.001$) over basal levels within 60 min (Fig. 5.2D). Gln did not activate any component of this cell signaling pathway (Fig. 5.2C).

Phosphorylation of RPS6K and RPS6 by arginine, leucine and glucose

The effects of Arg, Leu and glucose on the phosphorylation of RPS6K proteins were determined by immunofluorescence analyses (Fig. 5.3). In present study, we compared localization of total proteins and phosphorylated proteins. The oTr cells were incubated for 30 min with either Arg (0.2 mM), Leu (0.2 mM), Gln (2 mM) or glucose (4 mM). In untreated and glucose treated cells, total RPS6K was found primarily in the cytosol while low levels of phosphorylated RPS6K were detected in the nuclei (Fig. 5.3). The levels of nuclear phosphorylated RPS6K increased after treatment with Arg (Fig. 5.3). There was a slight, but insignificant, increase in phospho-RPS6K in Leu and Gln treated cells compared to control cells (Fig. 5.3). The intracellular location of total RPS6 protein was also determined by double-staining immunofluorescence (Fig. 5.4). Total RPS6 was detectable mainly in the cytoplasm of untreated and treated cells since RPS6 is the ribosomal protein typically activated through the mTOR pathway for mRNA translation in cytoplasm (302). After 30 min of treatment with Arg, Leu or glucose, phosphorylation of cytoplasmic RPS6 proteins increased significantly (Fig. 5.4).

Inhibition of effects of arginine, leucine and glucose by rapamycin and LY294002

To determine the cell signaling pathways mediating effects of Arg, Leu and glucose on AKT1, RPS6K and RPS6, oTr cells were pretreated with either pharmacological inhibitors of PI3K (25 μ M LY294002) or mTOR kinase activity (25 nM rapamycin) for 30 min. Induction of pRPS6K and pRPS6 by Arg, Leu and glucose was inhibited by both the PI3K inhibitor ($P < 0.001$) and rapamycin ($P < 0.005$) (Fig. 5.5) which indicated that activation of the RPS6K-RPS6 pathway by Arg, Leu and glucose is required for translational activation of both AKT1 and mTOR in the nutrient-induced cell signaling cascade.

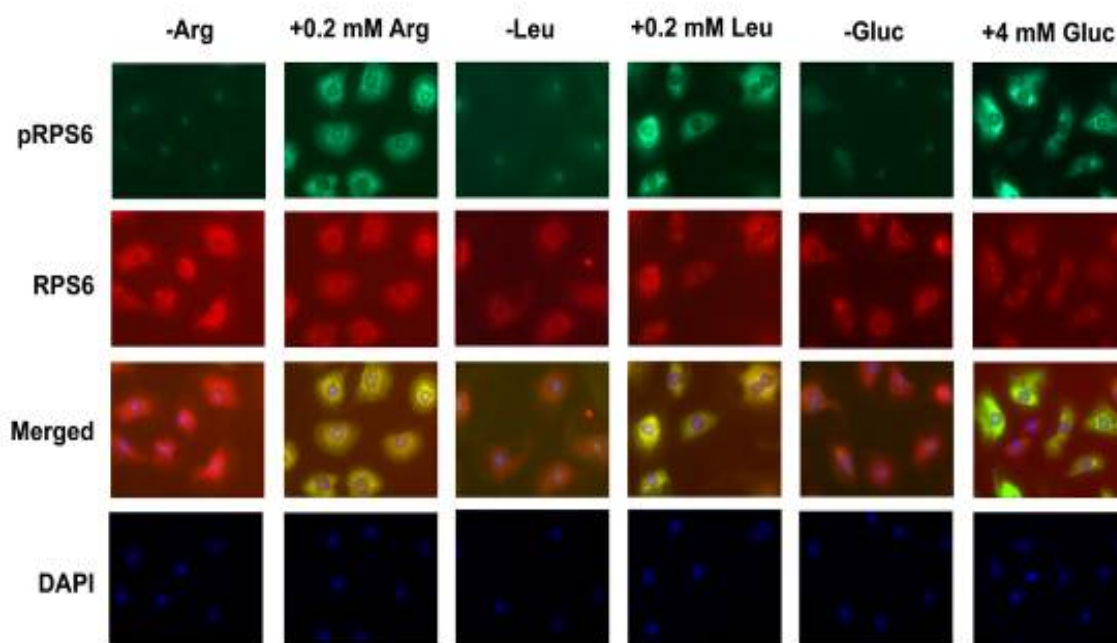


Fig. 5.4. Immunofluorescence localization of phosphorylated RPS6 protein in oTr cells. Co-localization of pRPS6 and total RPS6 after treatment with Arg (0.2 mM), Leu (0.2 mM) and glucose (4 mM) revealed that pRPS6 was significantly increased in cytoplasm of oTr cells treated with Arg, Leu and glucose compared to untreated cells.

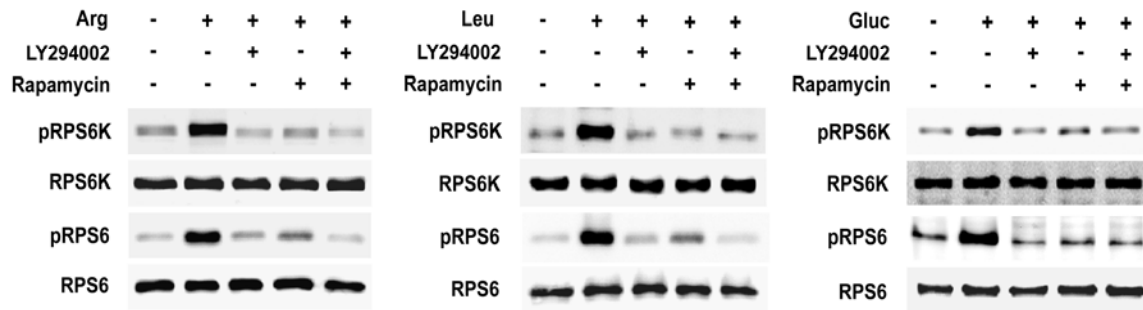


Fig. 5.5. Inhibition of RPS6K and RPS6 phosphorylation. Monolayers of 80% confluent oTr cells were serum-starved for 24 h, deprived of amino acids or glucose (Gluc) for 6 h and then pretreated with either 25 μ M LY294002 or 25 nM rapamycin for 30 min. After stimulation with Arg (0.2 mM), Leu (0.2 mM) or glucose (4 mM) for 30 min or 60 min, total cell lysates were subjected to 10% SDS-PAGE followed by western blotting. Both rapamycin and LY294002 inhibited increases in pRPS6K and RPS6. Blots were imaged to calculate the normalized values presented as levels of phosphorylated protein relative to total protein.

Effect of arginine, leucine, glutamine and glucose on cell proliferation and migration

Cell proliferation and migration assays were conducted to investigate biological effects of Arg, Leu, Gln and glucose on oTr cells. In the cell proliferation assays, treatment of oTr cells with Arg, Leu, Gln or glucose in customized medium containing 5% serum for 4 days increased oTr cell numbers by approximately 14-, 5-, 1.3- and 2.4-fold, respectively (Fig. 5.6). In addition, as illustrated in Fig. 5.7, Arg (0.2 mM), Leu (0.2 mM), Gln (0.5mM) and glucose (4 mM) increased migration of oTr cells about 3.3-, 2.3-, 1.4- and 1.7-fold, respectively.

Discussion

In the present study, specialized medium containing physiological concentrations of all other amino acids were used to mimic the *in vivo* state and cell deprived of select nutrients were used to determine their individual effects on signal transduction. Results of the present study provide the first detailed analyses of the action of individual amino acids and glucose on cell signaling pathways as well as selected biological effects on ovine trophectoderm cells. Results of the present study support our hypothesis (94, 95, 111, 297) that select nutrients can activate the mTOR cell signaling pathway to stimulate proliferation and migration of conceptus trophectoderm cells during the peri-implantation period of pregnancy in sheep.

Results of previous studies showed that early activation of mTOR signaling including AKT1, RPS6K and RPS6 by amino acids is required for the development of blastocysts and conceptuses (22, 96, 98, 287). In the present study, we first examined molecular mechanisms by which amino acids and glucose stimulate mTOR *in vitro* by determining effects on RPS6K phosphorylation, as an indicator of the activation of mTOR/RPS6K cell signaling pathway. Our results indicated that Arg and Leu, but not Gln, increased phosphorylation of RPS6K within 15 min and sustained this state of activation to 60 min. This finding supports several lines of evidence that Arg and Leu have the greatest effect on mTOR and RPS6K signaling in cells from the intestine and, in fact, most mammalian cells (303-308). Further, Arg- and Leu-induced phosphorylation of AKT1 and RPS6 was inhibited completely by both LY294002 (PI3K-AKT1 inhibitor) and rapamycin (mTOR inhibitor). In response to amino acid stimulation, RPS6K

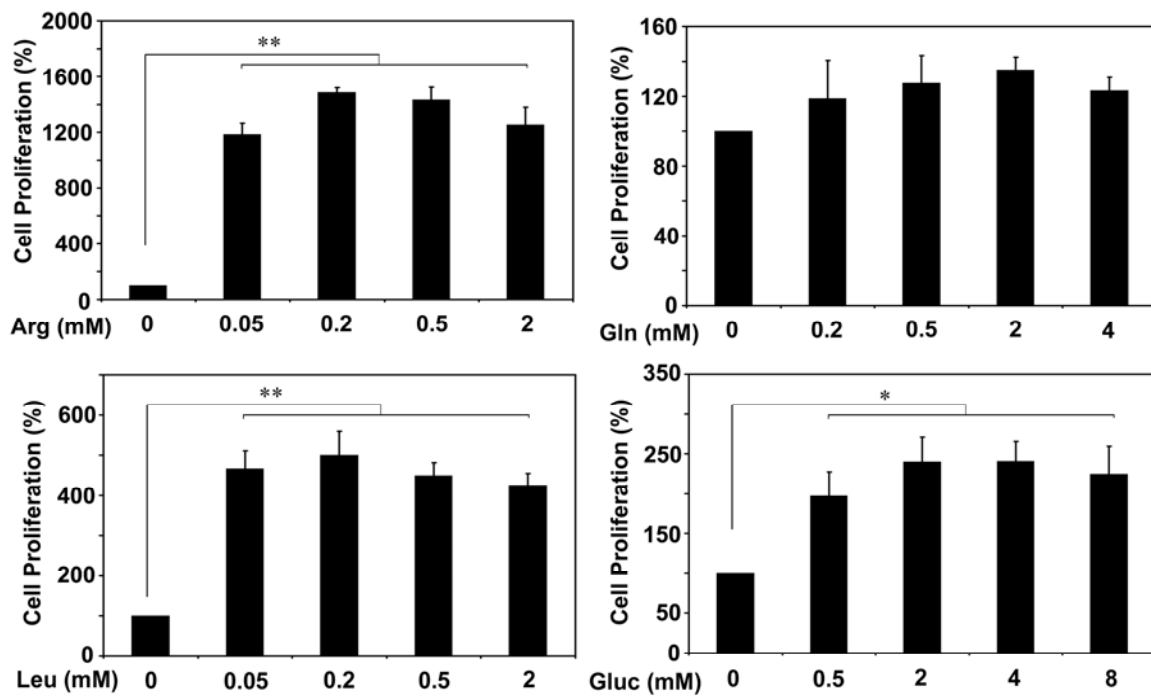


Fig. 5.6. Dose dependent effects of Arg, Leu, Gln and glucose (Gluc) on proliferation of oTr cells. The oTr cells were seeded at 30% confluency on microwells and cultured with indicated dose of Arg, Leu, Gln or glucose. Cell numbers were determined after 4 days of incubation, and data are expressed relative to nontreated control (100%). The *asterisk* (*) denotes an effect of treatment (*, $P < 0.01$; **, $P < 0.001$). All quantitative data are presented as LSMs with overall SEs. Arg (0.2 mM), Leu (0.2 mM) and glucose (4 mM) were most effective in stimulating proliferation of oTr cells by 15- ($p < 0.001$), 5.3- ($p < 0.001$) and 2.7- fold ($p < 0.01$), respectively, after 4 days while Gln (0.5 mM) did not have a significant effect.

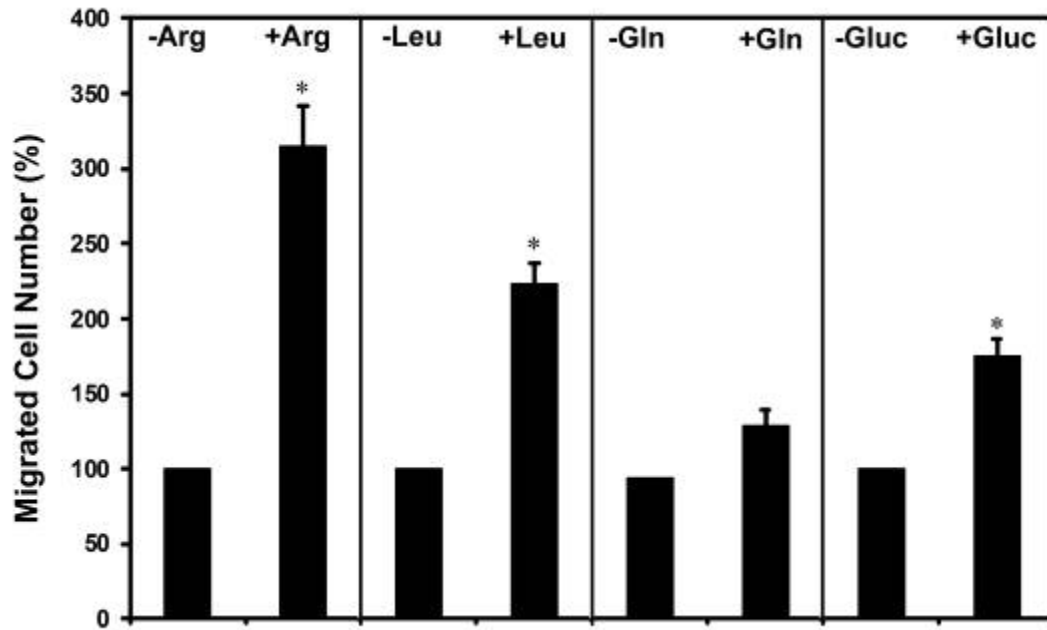


Fig. 5.7. Migration of oTr cells. The oTr cells were cultured in a transwell plate (n = three wells per treatment) and treated with Arg (0.2 mM), Leu (0.2 mM), Gln (0.5 mM) or glucose (Gluc, 4 mM). Cells grown in serum- and insulin-containing DMEM served as a positive control. Cell migration was determined after 12h treatment and expressed as LSMs \pm SE. Arg, Leu, and glucose stimulated oTr migration by 3.4-, 2.4- and 1.8- fold ($p < 0.01$) after 12 h of treatment, but cells treated with Gln (0.4 mM) did not show a significant change. The *asterisk* (*) denotes an effect of treatment ($P < 0.01$).

phosphorylates RPS6 proteins resulting in an increase in mRNA translation (108, 309, 310). In addition, immunoreactive pRPS6K protein translocated to and was particularly abundant in nuclei of oTr cells treated with Arg, which agreed with the spatial distribution of pRPS6K in nuclei of ovine trophectoderm and endoderm (268). Recently, the mTOR/RPS6K signaling cascade was demonstrated to exist in both the cytoplasm and nucleus (111, 311, 312). Phosphorylated RPS6 abundance in cells treated with Arg and Leu was significantly increased in cytoplasm, consistent with results from *in vivo* studies which indicated that pRPS6 was present in cytoplasm of conceptus trophectoderm (J. Kim and F. W. Bazer, unpublished data). Unlike Arg and Leu, Gln acts as a key regulator for amino acid controlled cell growth (313-315) and as a stimulator of mitogen-activated protein kinase and (mTOR)/RPS6K pathways, respectively, to enhance mucosal cell migration and wound healing in intestinal cells (316). Our finding that Gln did not affect phosphorylation of mTOR/RPS6K pathway proteins is consistent with that of Ban et al. (304). However, we cannot discount the possibility that Gln may be necessary for cell signaling cascades (317) because intracellular Gln is known to stimulate mTOR signaling by promoting the uptake of amino acids into cells (219, 316, 318, 319) and to be essential for the survival and growth of most cultured cells (320).

Glucose, a primary energy source, generates ATP to inactive AMPK (AMP activated protein kinase) which then leads to mTOR activation (321), but recent studies have indicated that the signals derived from glucose metabolites play important roles in the control of other cellular activities including phosphorylation of mTOR, which is essential for cell survival, growth and development (217, 220, 226, 322). However, little is known about signaling pathways stimulated by glucose in ovine trophectoderm during conceptus development during early pregnancy. In the present study, results indicated that glucose stimulates phosphorylation and activation of AKT1, RPS6K and RPS6 in oTr cells within 60 min and that this effect was inhibited completely by either LY294002 (PI3K-AKT1 inhibitor) or Rapamycin (mTOR inhibitor). These results indicate that glucose-induced mTOR activation in oTr cells is mediated through PI3-AKT cell signaling. It has been proposed that glucose stimulation of mTOR was mediated by formation of glutamine-6-phosphate in the GFAT reaction (21) and activity of GFAT is regulated by glucose uptake and AMP-activated protein kinase (AMPK) (323, 324).

Glucose also stimulated oTr cell proliferation (Fig 6), consistent with results from studies with human trophoblasts in that glucose-induced trophoblast cell proliferation is mediated through mTOR activation (21). Further, immunoreactive pRPS6 proteins were abundant in cytoplasm of cells treated with glucose, similar to effects mediated by Arg and Leu. Thus glucose stimulates the mTOR signaling pathway through AKT for protein translation and proliferation which supports rapid growth of ovine trophoblast cells during the peri-implantation period.

In addition to activation of cell signaling pathways, Arg and Leu increased proliferation and migration of oTr cells (Figs. 5.6 and 5.7). Our laboratory has also identified that several candidate molecules including galectin 15, SPP1 and IGF2 that play a critical role in implantation and increase migration of oTr cells, but not proliferation (268, 300). The effects of Arg and Leu on cell proliferation are striking and indicate their physiological importance for conceptus growth. We previously reported that the expression of NOS, GCH1, and ODC1 in ovine uterine endometria and conceptuses increased during peri-implantation when the conceptus is undergoing rapid growth, attachment and establishment of pregnancy (325). Thus, results of the present studies strongly support the hypothesis that selected amino acids and glucose transported into the uterine lumen by uterine LE and GE stimulate proliferation and migration of conceptus trophectoderm via activation of the AKT1-FRAP1-RPS6K-RPS6 cell signaling cascade.

In conclusion, amino acids and glucose activate the AKT1-mTOR-RPS6K-RPS6 cell signaling cascade to affect activation of developmentally-regulated genes affecting cell proliferation and migration in ovine conceptuses during the peri-implantation period. These results provide important insights into the mechanisms by which select nutrients regulate conceptus development during the peri-implantation period of pregnancy in sheep.

CHAPTER VI

ARGININE STIMULATES PROLIFERATION OF OVINE TROPHECTODERM CELLS THROUGH FRAP1/mTOR-RPS6K-RPS6 SIGNALING CASCADE AND SYNTHESIS OF NITRIC OXIDE AND POLYAMINES

Introduction

L-Arginine (Arg) is a common substrate for nitric oxide (NO) and polyamine syntheses via NO synthase (NOS) and ornithine decarboxylase (ODC) (326), which are important for embryonic survival, fetal-placenta growth and development (119, 205), as well as trophoblast outgrowth and cell migration (96, 327). Nitric oxide is a major angiogenic factor (328) and plays a role in regulating uteroplacental-fetal blood flows that are essential for the transfer of nutrients and oxygen from mother to fetus (203, 327). The polyamines, putrescine, spermidine and spermine, are required for DNA and protein synthesis and for proliferation and differentiation of mammalian cells (204, 329). In addition, Arg mediates central metabolic pathways for nutrient utilization and protein synthesis through FKBP12-rapamycin complex-associated protein 1 (mTOR, also known as FRAP1) and NO cell signaling pathways (22, 96, 209).

Uterine endometrial and fetuses from many species, including mice (330, 331), rats (332), sheep (91, 197, 325, 333), mares (334), humans (335) and pigs (336, 337), have the capacity to catabolize Arg, due to the presence of NOS and ODC, to increase production of NO and polyamines during gestation. In contrast, inhibition of NO synthesis by NOS inhibitors such as L-NAME in rats or the absence of NO synthesis in eNOS-knockout mice restricts fetal growth (338, 339). Also, inhibition of polyamine synthesis prevents mouse embryogenesis and impairs fetal growth (329). This evidence suggests that Arg-dependent metabolic pathways, including synthesis of NO and polyamines, are critical for conceptus growth and development in mammalian species.

We reported that Arg increased 10-fold in uterine flushings from ewes during the peri-implantation period of pregnancy (95) and that cationic amino acid transporters, such as SLC7A1 and SLC7A2, were expressed in trophoctoderm and endoderm of conceptuses, as well as uterine luminal (LE) and glandular (GE) epithelia in response to progesterone and interferon tau (IFNT) in sheep (297). In ruminants, which have a synepitheliochorial

placenta and a protracted preimplantation period, Arg is transported into the uterine lumen to support conceptus development; however, little is known about the mechanisms whereby Arg stimulates ovine trophoblast hyperplasia and rapid elongation during the peri-implantation period of pregnancy. Therefore, the objectives of the present study were to determine whether Arg stimulates trophoblast cell proliferation, and whether increased production of NO and polyamines is involved in mediating cell proliferation.

Materials and Methods

Cell culture

Mononuclear ovine trophectoderm (oTr) cells from Day 15 conceptuses were isolated and cultured as described previously (181, 268). One cell line, referred to as oTr1, was cultured in DMEM-F12 that included 10% FBS, 50U penicillin, 50 µg streptomycin, 0.1 mM each for non-essential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, and 0.7 µM insulin. When the density of cells in dishes reached about 80% confluence, they were passaged at a ratio of 1:3, and frozen stocks of cells were prepared at each passage. For experiments, monolayer cultures of oTr cells (between passages 9 and 13) were grown in culture medium to 80% confluence on 100-mm tissue culture plates. Cells were serum starved for 24 h in customized medium, deprived of arginine for 6 h, and then treated with Arg in a dose- and time-dependent manner. For each experiment, this design was replicated three independent times.

Immunofluorescence

The effects of Arg on phosphorylation of RPS6K and RPS6 were determined by immunofluorescence microscopy as previously described (234, 264). Briefly, oTr cells were grown in Lab-Tek four-well chamber slides (Nalgene Nunc International, Rochester, NY) and treated with 0 or 0.2 mM Arg for 30 min. Cells were then fixed for 10 min in paraformaldehyde, rinsed, blocked in 5% normal goat serum, and incubated in primary antibody overnight at 4°C. Rabbit anti-p4EBP1 antibody (Catalog number 9451, Cell Signaling Technology, Danvers, MA) was used at a 1: 200 dilution, and goat anti-rabbit IgG Alexa 488 (Chemicon) for 1 h at room temperature. Cells were then washed and overlaid with Prolong Gold Antifade with DAPI. Slides were stored at 4°C in the dark.

before immunofluorescence analysis. Fluorescence images of cells after treatment were captured using a Zeiss Stallion Dual Detector Imaging System with Intelligent Imaging Innovations Software (Carl Zeiss, Thornwood, NY).

Proliferation assay

The oTr cells were subcultured into 24-well plates (20% confluent) in growth medium until the monolayer reached up to 50% confluency and then switched to serum- and insulin-free customized medium. After starvation for 24 h, cells were deprived of Arg for 6 h and then Arg was added to each well (n=3 wells per treatment) that included combinations of either: (a) serum- and insulin-free specialized medium, a negative control; (b) Arg 0 to 200 μ M with 5% serum; or (c) medium containing 10% fetal calf serum as a positive control. The media were changed every 2 days and treated cells were maintained for 4 days. Cell numbers were determined as described previously (236, 299). Briefly, DMEM was removed from cells by vacuum aspiration, and cells were fixed in 50% ethanol for 30 min, followed by vacuum aspiration of the fixative. Fixed cells were stained with a Janus Green B in PBS (0.2% w/v) for 3 min at room temperature. The stain was immediately removed using a vacuum aspirator, and the whole plate was sequentially dipped into water and destained by gentle shaking. The remaining water was removed by shaking, stained cells were immediately lysed in 0.5N HCl, and absorbance readings were taken at 595 nm using a microplate reader. As described previously (299), cell numbers were calculated from absorbance readings using the following formula: cell number = (absorbance – 0.00462)/0.00006926. The entire experiment was independently repeated three times with different batches of oTr cells between passages 7 and 10.

Migration assay

Migration assays were conducted with oTr cells as described previously (268, 300). Briefly, oTr cells (50,000 cells per 100 μ l serum and insulin-free DMEM) were seeded on 8 μ m pore Transwell inserts (Corning Costar #3422, Corning, NY). Treatments were identical to those previously described for the proliferation assay (n=3 wells per treatment). After 12 h, cells on the upper side of the inserts were removed with a cotton swab. For evaluation of cells that migrated onto the lower surface, inserts were fixed in 50% ethanol for 5 min. The transwell membranes were then removed, placed on a glass slide with the side containing cells facing up, overlaid with Prolong antifade mounting reagent with DAPI (Invitrogen-Molecular Probes, Eugene, OR), and cover slipped. Cells that had migrated were systematically counted using a Zeiss Axioplan 2 fluorescence microscope with AxioCam HR digital camera and Axiovision 4.3 software (Carl Zeiss Microimaging, Thornwood, NY). The entire experiment was repeated at least three times with different batches of oTr cells between passages 7 and 10.

Protein synthesis and degradation

Protein synthesis and degradation were measured in oTr cells cultured with radiolabeled Phe as previously described with minor changes (340). Briefly, oTr cells were incubated with Arg in customized medium at concentrations of 0 and 0.2 mM for 4 days and then switched to 2 ml medium containing 0.8 μ Ci L-[ring-2,4-³H]phenylalanine (American Radiolabeled Chemicals) for 2 h and maintained at 37°C in an incubator. Following incubation, the media were collected and cells washed three times with 2 ml ice-cold PBS. The cell pellet was mixed with 2 ml of 2% trichloroacetic acid (TCA) (341). Cells in each well were scraped and then the whole solution was collected and centrifuged. The cell pellet was washed with 5 ml of 2% TCA and dried in air at 37°C. After the pellet was dissolved in 0.5 ml of 1 M NaOH, 0.4 ml of the solution was transferred to a 20 ml scintillation vial containing 15 ml of Hionic Fluor Scintillation cocktail (PerkinElmer, MA, USA). The ³H-Phe radioactivity was determined by a liquid scintillation counter after samples had stood overnight at room temperature. Specific activity of ³H-Phe in medium was used to calculate rate of protein synthesis in cells.

For determining protein degradation, oTr cells were cultured for 3 days in 2 ml of Arg-free medium and then in doses from 0 to 2 mM Arg. Beginning on day 4, cells were cultured for 24 h in 2 ml of Arg free medium containing 0.1 mM L-phenylalanine plus L-[³H]-Phe (0.8 µCi/well) and various concentrations of Arg. After the 24 h culture to label cellular proteins, cells were washed three times with 2 ml Arg-free medium containing 1 mM L-phenylalanine to deplete intracellular free [³H]-Phe (341). The cells were then cultured for 2 h in 2 ml medium containing 1 mM L-Phe and various concentrations of Arg. At the end of the 2 h culture period, the medium was collected, cells were washed with 2 ml ice-cold PBS, and 2 ml of 2% TCA was added to each well. The whole TCA extract was collected into a 15 ml tube and centrifuged at 3,000g for 5 min. The supernatant fluid was removed and the pellet was washed three times with 5 ml of 2% TCA and dried in air at 37°C. After the pellet was dissolved in 0.5 ml of 1 M NaOH, 0.4 ml of the solution was transferred to a 20 ml scintillation vial containing 15 ml Hionic Fluor Scintillation cocktail for measurement of [³H]-Phe. For determining [³H]-Phe released from prelabeled proteins into the culture medium, the collected medium was centrifuged at 3,000g for 2 min to remove dead cells. An aliquot (1 ml) of the supernatant fluid was transferred to a 15 ml tube containing 2 ml of 2% TCA. After the tubes were centrifuged at 3,000g for 5 min, all the supernatant fluid was transferred to a 20 ml scintillation vial containing 15 ml Hionic Fluor Scintillation cocktail for measurement of [³H]-Phe. The percentage of protein-bound [³H]-Phe released into culture medium (namely [³H]-Phe in medium/[³H]-Phe in cell proteins x 100) was calculated to indicate protein degradation in oTr cells.

Preparation of ovine conceptuses

Using procedures described previously (342, 343), conceptuses from Day 16 pregnant ewes (coincident with maximal production of IFNT by the conceptuses) were recovered by flushing each uterine horn with 20 ml minimal essential medium (MEM). The conceptuses were initially cultured for 6 h in Arg-free medium and then cultured in designated doses of Arg for 18 h at 37°C with rocking in a 50% O₂, 45% N₂, 5% CO₂ atmosphere. The culture medium was collected after centrifugation and protease inhibitors (Complete EDTA-free Protease Inhibitor Cocktail; Roche Diagnostics, Indianapolis, IN) were added. The culture supernatant was diluted and stored at 4°C until analyzed. Conceptuses were homogenized, the homogenate assayed for protein and then stored for analyses.

Western blot analyses

Whole cell extracts and immunoblot assays were prepared and performed as described previously (234, 268). To harvest total cellular protein for western blot analyses, oTr cells were rinsed with cold PBS and lysed by incubation in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na₃VO₄, 0.2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 30 mM Na₄P₂O₇, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 30 min at 4°C. Cell lysates were passed through a 26-gauge needle and clarified by centrifugation (16,000 x g, 15 min, 4°C). The protein content was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured, separated using SDS-PAGE, transferred to nitrocellulose and western blotting performed as described previously (268) using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and X-OMAT AR X-ray film (Kodak, Rochester, NY) according to manufacturer's recommendations. Immunoreactive proteins were detected by using rabbit anti-mouse polyclonal p-mTOR IgG (Catalog numbers 2971) at a 1:1,000 and mTOR IgG (Catalog numbers 2972) at a 1:2,000 dilution and 10% SDS-PAGE gel, rabbit anti-human polyclonal p-RPS6K IgG (Catalog number 9204) at 1:1,000 and RPS6K IgG (Catalog number 9202) at 1:2,000 dilution and 10% SDS-PAGE gel, and rabbit anti-human polyclonal phospho-4EBP1 IgG (Catalog number

9451) and 4EBP1 IgG (Catalog number 9644) at a 1:1,000 dilution and 12% SDS-PAGE gel. Immunoreactive IFNT was detected using primary rabbit anti-ovine IFNT serum at a 1:5000 dilution. Rabbit anti-ovine IFNT serum was generated by immunizing rabbits with recombinant ovine IFNT (344). As a loading control, mouse anti-alpha tubulin (TUBA) IgG (Catalog number; T5168; Sigma, St. Louis, MO) was used. Multiple exposures of each western blot were performed to ensure linearity of chemiluminescent signals. Western blots were quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA).

Statistical analyses

Results are expressed as mean \pm SEM. The statistical analysis was performed by one-way or two-way ANOVA using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). P values of < 0.05 were taken to indicate significance.

Results

Effects of D-arginine on oTr cell proliferation and phosphorylation of signal transduction

To identify biological effects and availability of D-Arg in oTr cells, cell proliferation assays were conducted. It was determined that treatment of oTr cells with D-Arg had no effect on oTr cell proliferation, while L-Arg at 0.2 mM increased cell numbers by approximately 15-fold ($P < 0.001$; Fig. 6.1A). D-Arg did not affect the phosphorylation status of RPS6K and RPS6 signaling molecules (Fig. 6.1B).

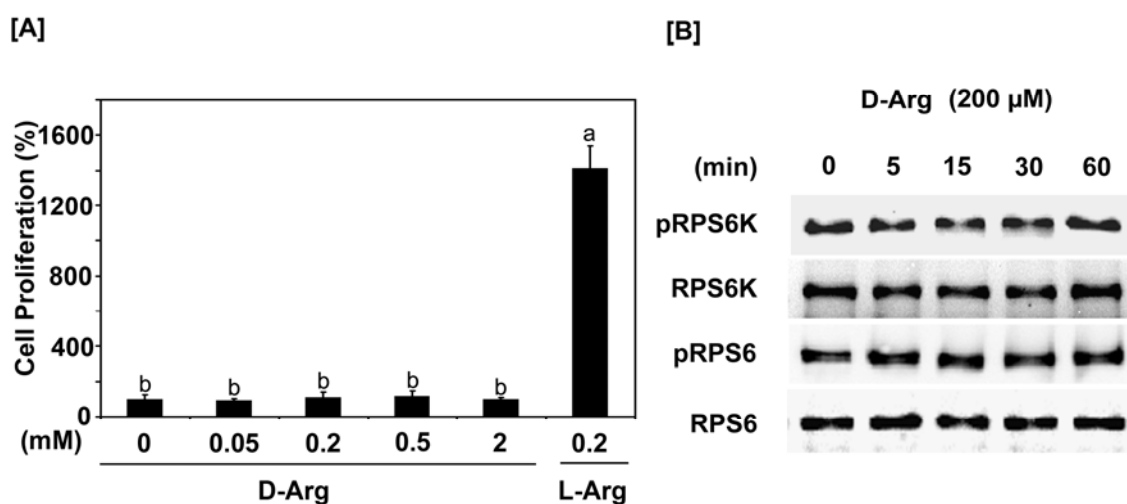


Fig. 6.1. Analysis of oTr cell proliferation and the phosphorylation of signal transduction proteins in response to D-arginine (D-Arg). A. The oTr cells were seeded at 30% confluency in microwells and cultured in Arg-free customized medium containing 0, 0.05, 0.2, 0.5 and 2 mM D-Arg which did not stimulate cell proliferation; however, oTr cells in medium containing 0.2 mM L-Arg had a 15-fold increase in proliferation after 96 h of culture. Data are expressed as means \pm SEM (n=8). Means sharing different letters differ ($P < 0.001$). B. Monolayers of 80% confluent oTr cells were serum-starved for 24 h, deprived of Arg for 6 h and then treated with D-Arg at 200 μ M for the indicated periods of time. Treatment with D-Arg did not alter levels of phosphorylated protein compared to control values.

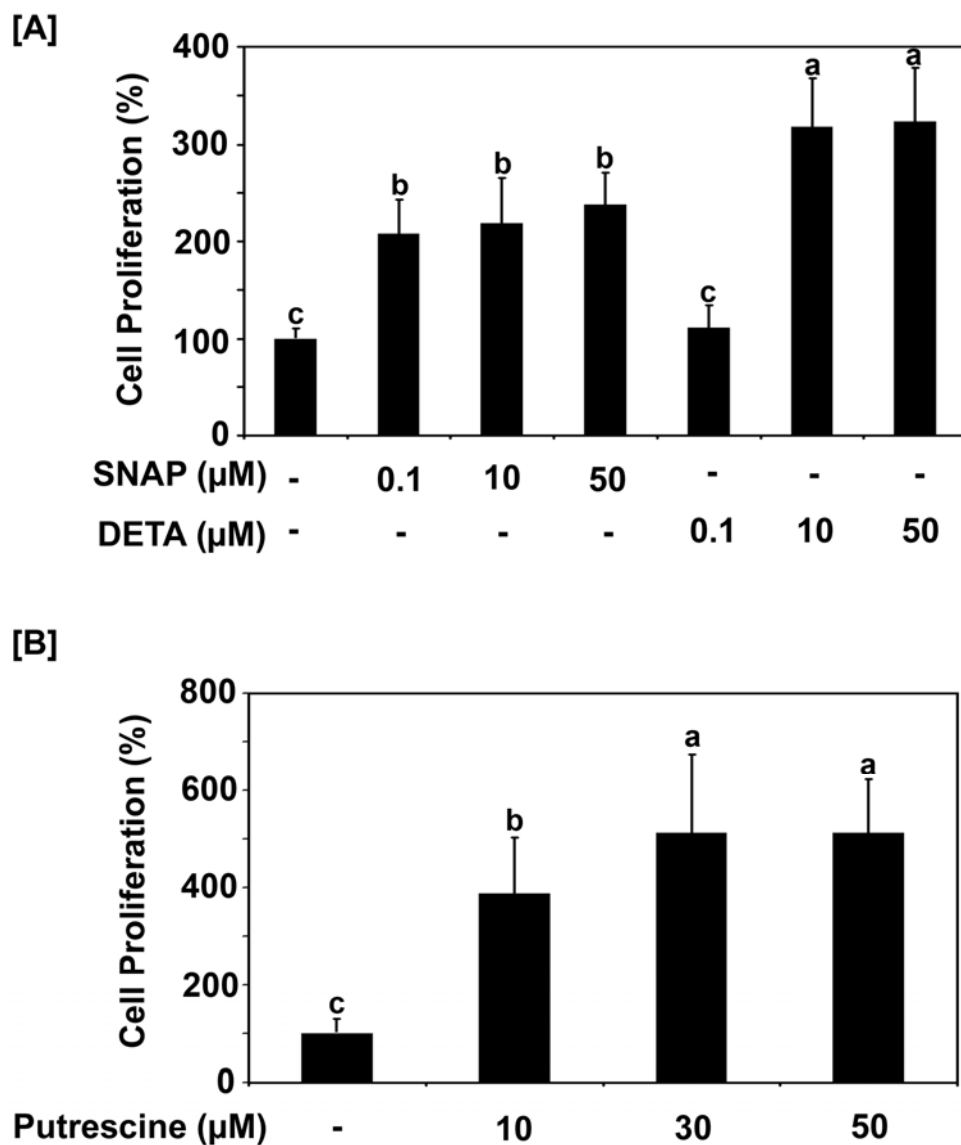


Fig. 6.2. Proliferation of oTr cells in response to NO donors and putrescine. A. NO donors (SNAP and DETA) increased proliferation of oTr cells. B. Polyamines (putrescine) increased proliferation of oTr cells. The oTr cells, seeded on microwells, were cultured in Arg-free customized medium containing either putrescine, SNAP or DETA at the indicated doses. The cell numbers, determined after 96 h incubation, are expressed as means \pm SEM ($n=8$) and indicated that both NO donors and putrescine increased cell proliferation. Means with different letters differ ($P<0.05$).

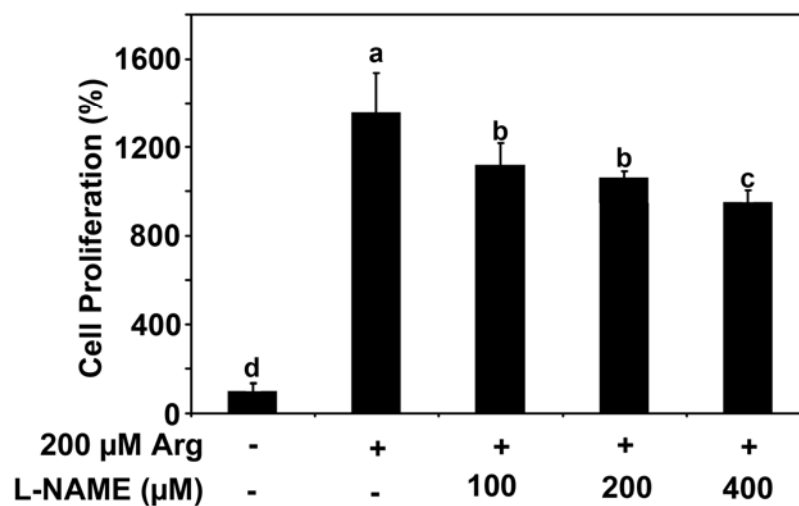
Effect of NO donors and polyamine precursor on oTr cell proliferation

To evaluate the direct effects of NO and polyamine on cell proliferation, cells were treated with either the NO donors, *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and diethylenetriamine NONOate (DETA) or polyamine precursor, putrescine. Both SNAP and DETA enhanced proliferation of oTr cells ($p < 0.01$); however, effects of DETA (10 or 50 μM) were greater than for SNAP ($p < 0.01$) (Fig. 6.2A). Putrescine also stimulated cell oTr proliferation at 30 μM ($p < 0.001$) (Fig. 6.2B).

Inhibitory effects of L-NAME and nor-NOHA on oTr cell proliferation

Treatment of oTr cells with either L-NAME or nor-NOHA resulted in a concentration-dependent decrease in proliferation (Fig. 6.3A and 6.3B). In the presence of 200 μM nor-NOHA, proliferation was reduced to approximately 60% of values for cells treated only with Arg ($P < 0.001$). The NOS inhibitor, L-NAME, also inhibited proliferation of oTr cells, but the effect was less than that for cells treated with 10 μM nor-NOHA (Fig. 6.3B).

[A]



[B]

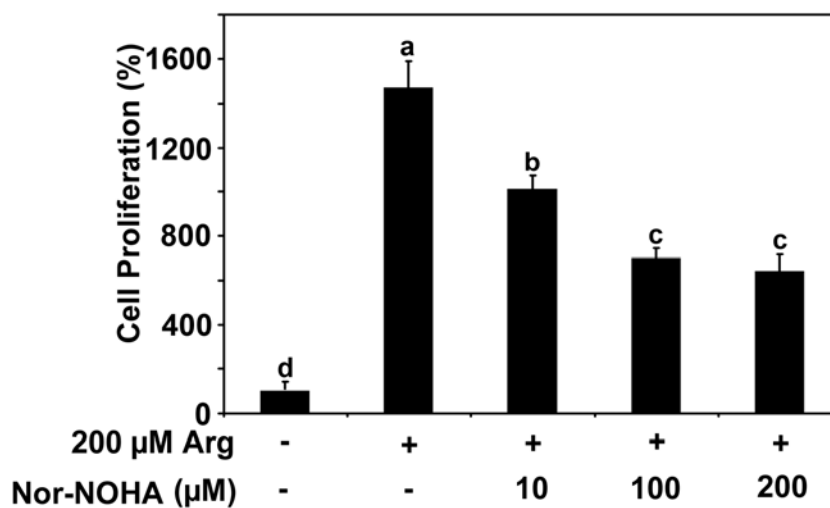


Fig. 6.3. Inhibition of Arg-induced proliferation of oTr cells by L-NAME and nor-NOHA. The oTr cells were seeded on microwells cultured in 0.2 mM Arg in customized medium with either L-NAME or nor-NOHA at the indicated doses. Based on cell numbers determined after 4 days incubation and expressed as means \pm SEM ($n=8$), both L-NAME and nor-NOHA decreased cell proliferation. Means with different letters differ ($P<0.001$).

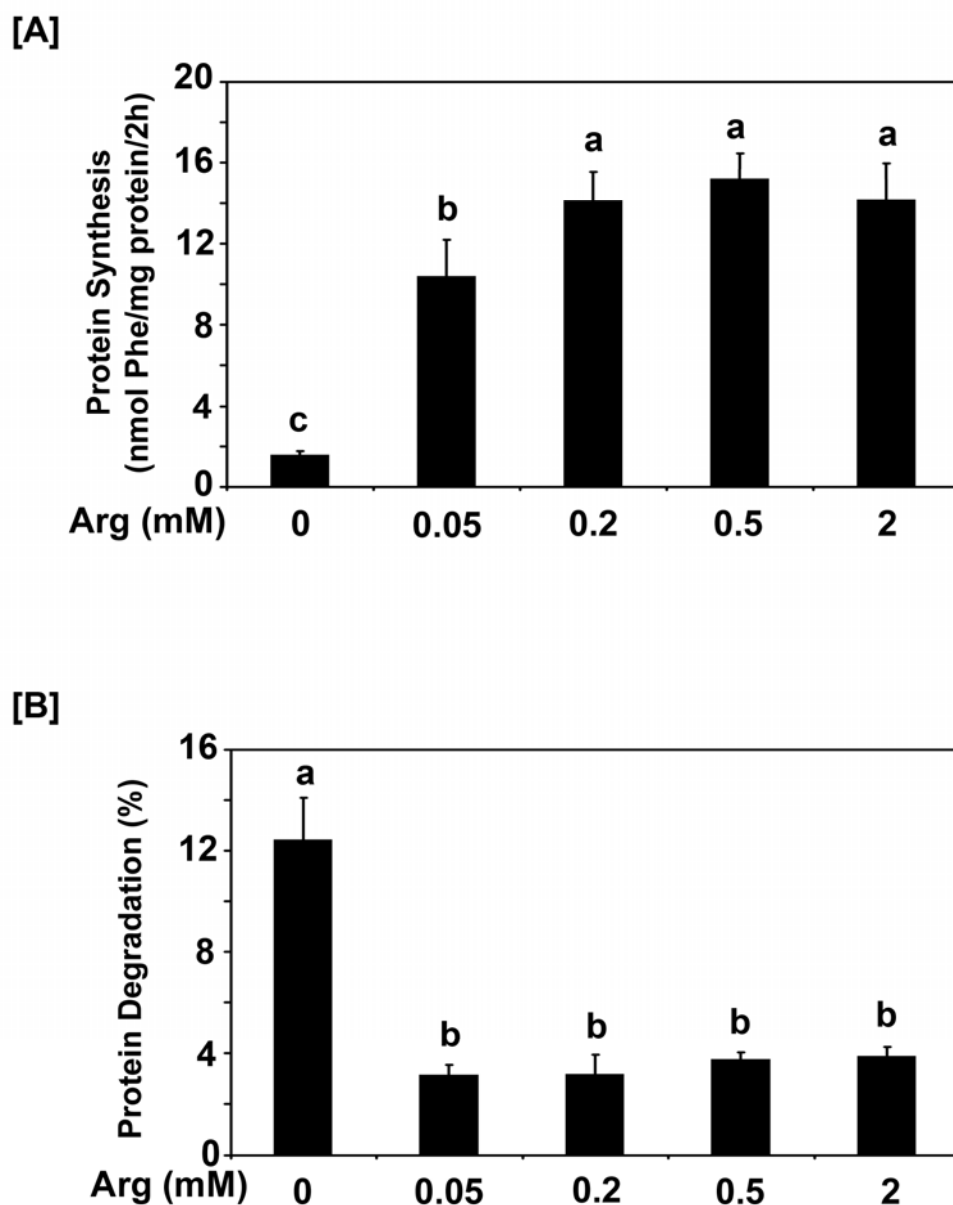


Fig. 6.4. Protein synthesis and protein degradation in oTr cells treated with Arg. The oTr cells were cultured in Arg-free customized medium containing 0, 0.05, 0.2, 0.5 and 2 mM Arg for 96 h and then placed in medium containing Arg. Results, expressed as means \pm SEM ($n=8$), indicated that Arg stimulated protein synthesis (Panel A) and inhibited protein degradation (Panel B) by oTr cells. Means with different letters differ ($P<0.001$).

Effect of arginine on protein synthesis and degradation in oTr cells

Cell growth is regulated by a balance between protein synthesis and degradation. Therefore, we compared the *in vitro* rates of protein synthesis and degradation based on incorporation of [³H]-Phe for 2 h. Arg-treated cells had a significant increase in protein synthesis that ranged from 5- to 8- fold higher than for control cells in medium that lacked Arg (Fig. 6.4A). Furthermore, despite the short period of culture, protein degradation in control oTr cells was inhibited by L-Arg (Fig. 6.4B).

Effect of arginine on phosphorylation of mTOR, RPS6K and 4EBP1 proteins

Arg had no effect ($P>0.10$) on total MTOR, RPS6K or 4EBP1 proteins in oTr cells. Compared to the control, addition of 0.05 and 0.2 mM Arg, respectively increased levels ($P<0.01$) of p-mTOR (73% and 203%), p-RPS6K (201% and 207%) and p-4EBP1 (52% and 204%) (Fig. 6.5).

Production of IFNT in response to arginine by explants cultures of ovine conceptuses

IFNT, a protein produced by ovine conceptus trophectoderm, is the primary maternal recognition of pregnancy signal in ruminants (345). To determine whether Arg stimulates synthesis of proteins by ovine conceptuses, amounts of IFNT were quantified in culture medium by western blotting. Arg (0.2 mM) increased ($P<0.05$) IFNT production and secretion into culture medium as compared to culture medium lacking Arg (Fig. 6.6). For a loading control, an equal volume of conceptus lysate was loaded and probed with α -tubulin.

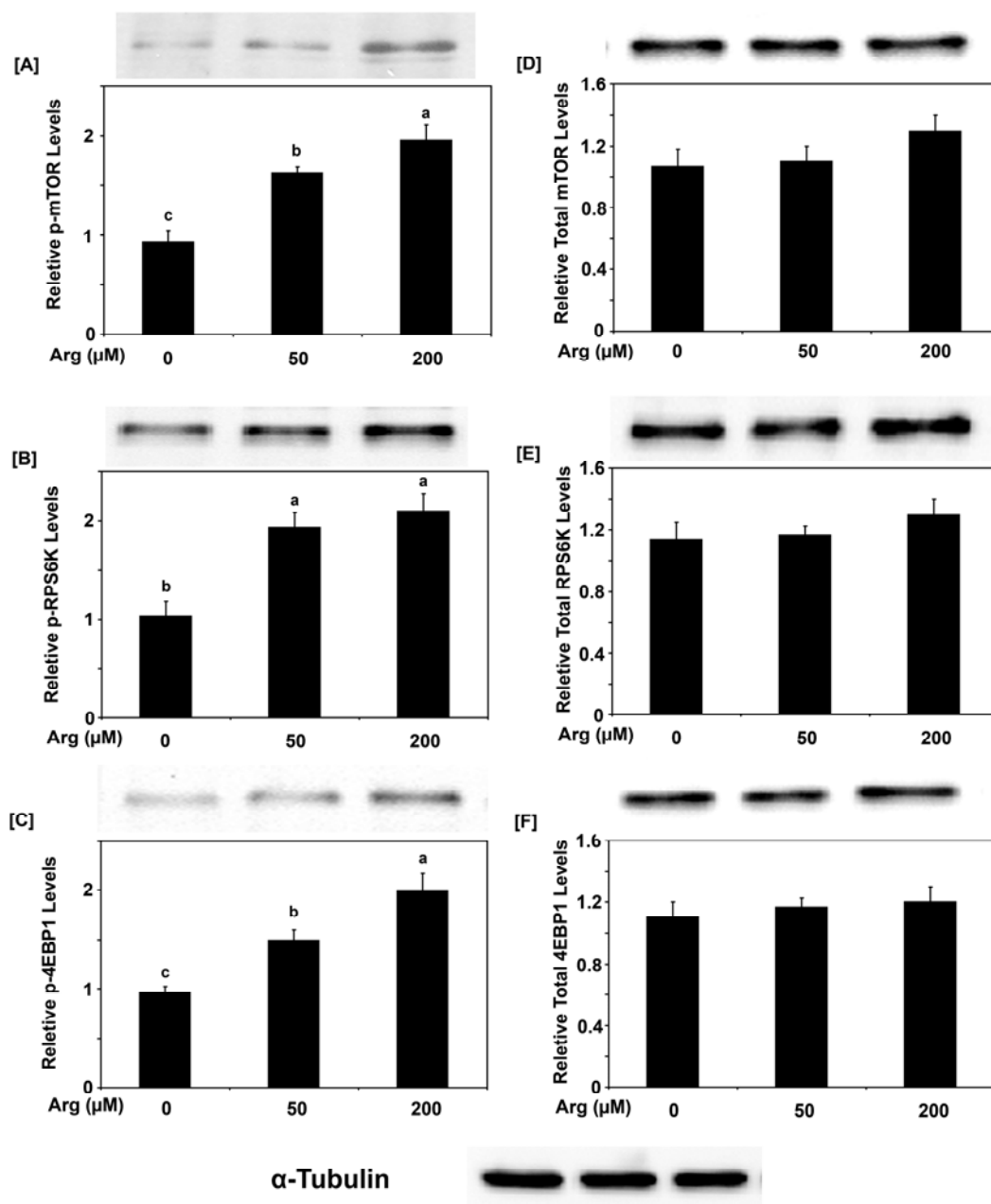


Fig. 6.5. Relative increases in phosphorylation of select proteins in the mTOR cell signaling pathway. oTr cells were cultured in Arg-free customized medium containing 0, 0.05 and 0.2 mM Arg for 96 h. Western blotting was used to calculate normalized values relative to total α -tubulin protein. Treatment of oTr cells with Arg increased abundance of phosphorylated mTOR, RPS6K and 4EBP1 at 0.05 and 0.2 mM, compared with control values, but Arg had no effect on total protein values for these cell signaling proteins. Data are expressed as means \pm SEM (n = 4). Means with different letters differ ($P < 0.001$).

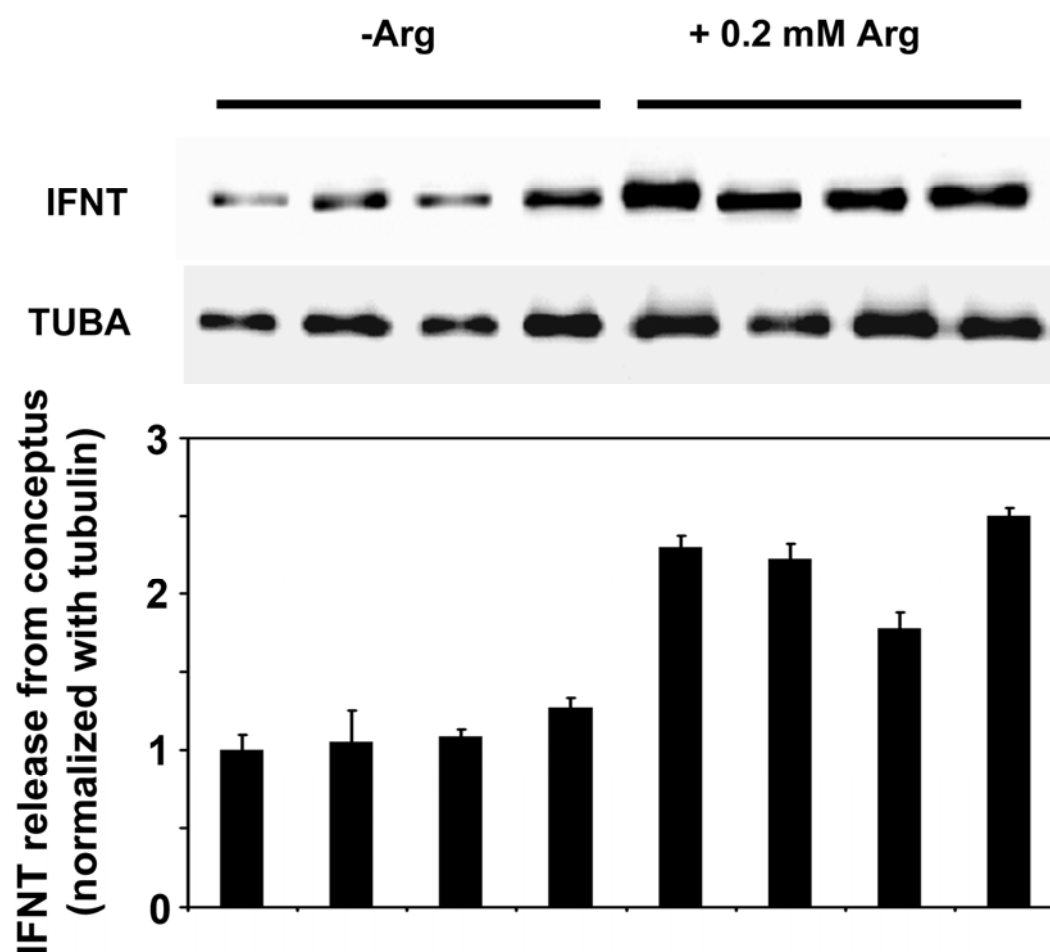


Fig. 6.6. The effect of Arg on synthesis and release of IFNT from explant cultures of ovine conceptuses. Ovine conceptuses obtained on Day 16 of pregnancy were washed, dissected into equivalent pieces and placed in explant culture medium. Conceptuses were cultured in medium lacking Arg for 6 h and then incubated in medium containing 0.2 mM Arg for 18 h. INFT production was normalized to values for amount of alpha tubulin (TUBA). Arg-treated conceptuses released more IFNT than those in control medium ($P<0.05$). Data are expressed as means \pm SEM ($n=4$). Means with different letters differ ($P<0.05$).

Stimulation of oTr cell migration by NO

This experiment determined if the effect of Arg on migration oTr cells required NO synthesis by incubating oTr cells with SNAP or DETA which are both NO donors. Both SNAP and DETA stimulated migration by 1.5- and 1.8- fold ($P < 0.01$) (Fig. 6.7). However, Arg at 200 μ M was more effective than either SNAP or DETA in stimulating migration of oTr cells ($P < 0.01$).

Effects of arginine on phosphorylation of 4EBP1 and its cellular localization

The 4EBP1 protein binds directly to eIF4E to inhibit complex assembly for translation initiation. Phosphorylation of 4EBP1 results in its release from eIF4E to allow translation of mRNAs to proceed. Arg significantly increased phosphorylation of RPS6K and RPS6, which are key components of the classical mTOR translation pathway (Figs. 6.7A, 6.7B and 6.7C). Therefore, we investigated whether 4EBP1 is present and phosphorylated in oTr cells and its intra-cellular localization. Results indicated that Arg increased p-4EBP1 in nuclei of oTr cells and that this phosphorylation was inhibited by rapamycin (Fig. 6.8), which suggests that phosphorylation of 4EBP1 occurs via the mTOR cell signaling pathway.

Discussion

Amino acids influence fetal-placental growth and development; therefore, providing adequate amounts of these nutrients to the conceptus is critical to pregnancy. It has been reported that Arg supplementation ameliorated intrauterine growth restriction in women (346, 347) as well as enhanced fetal survival and growth in rats (201) and gilts (202). Arg is required for protein synthesis and it is a precursor of many metabolically important molecules, including proline, ornithine, polyamines, and NO (202, 205). Evidence from studies of pigs and sheep indicates that increased availability of Arg in fetal fluids is associated with high rates of synthesis of NO and polyamines during early gestation when placental growth is most rapid (91, 333, 336, 337). NO synthase is required for the synthesis of NO from Arg, whereas arginase catalyzes the conversion of Arg to urea and to ornithine which is the principal precursor for production of polyamines (348). Impaired NO and polyamine production from Arg is not only

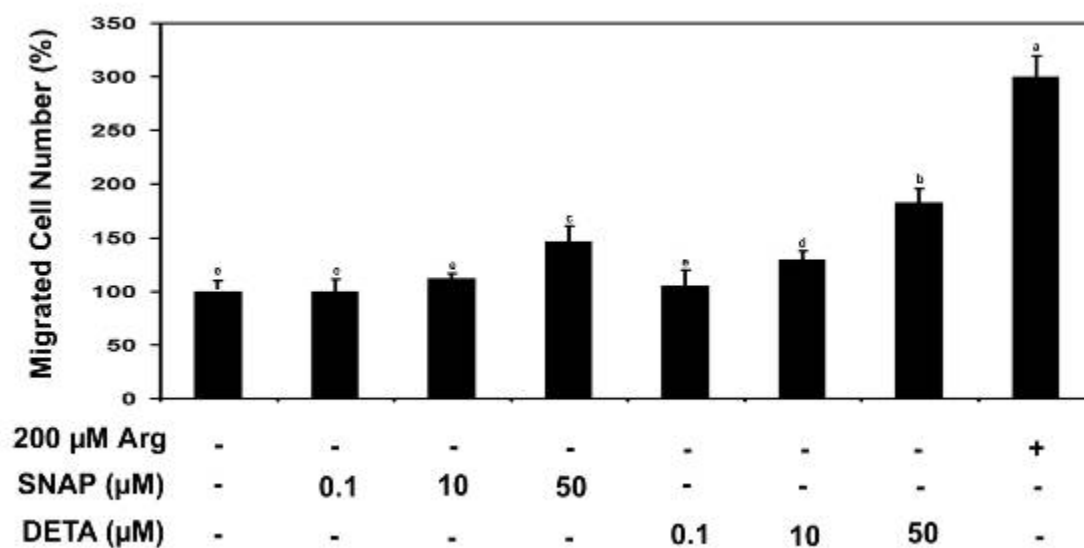


Fig. 6.7. Migration of oTr cells by NO donors. The oTr cells were cultured in a transwell plate (n = three wells per treatment) and treated with either SNAP or DETA. Cells grown in 0.2 mM Arg containing DMEM served as a positive control. DETA at 50 μ M was effective in stimulating oTr migration after 12 h of treatment. Data are expressed as means \pm SEM (n=4). *a-e* Means with different letters differ ($P<0.01$).

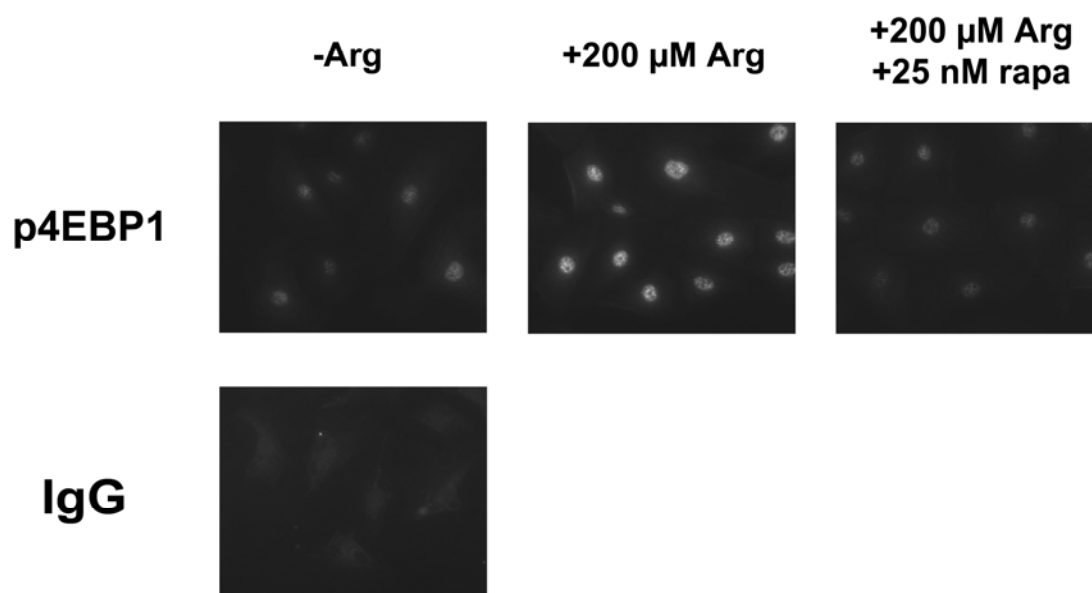


Fig. 6.8. Immunofluorescence localization of phosphorylated 4EBP1 protein in oTr cells. Phosphorylated 4EBP1 was observed in nuclei after treatment with 0.2 mM Arg, and this nuclear translocation was inhibited by pretreatment with rapamycin (rapa). Rabbit IgG served as a negative control.

responsible for embryonic death and inhibition of trophoblast outgrowth, but also fetal defects and failure of implantation which reflect the importance of Arg-dependent metabolic pathways in conceptus growth and development (197, 349-351). It has been reported that the mTOR cell signaling pathway is a key regulator of metabolic actions of Arg (305, 306). However, little is known about the molecular mechanisms whereby Arg stimulates proliferation of cells of ovine conceptuses. These studies with cultured oTr cells revealed that Arg stimulates the phosphorylation of proteins in the mTOR signaling pathway including RPS6K and RPS6 (J. Kim and F.W. Bazer, unpublished observation). Polyamine biosynthesis is closely related to DNA synthesis (352) and requires actions via a number of cellular pathways relevant for cell proliferation (204), but these molecular mechanisms are not fully defined. NO can activate mTOR and increase synthesis of polyamines (96). Indeed, activation of mTOR signaling represents an important step in the mechanism of action of NO and polyamines (353). A large body of evidence indicates that NO is involved in pregnancy through its functions as a vasodilator (327), an important regulator of embryogenesis (205) and trophoblast functions such as implantation, as well as differentiation, motility, invasion and apoptosis of cells (354-356). Evidence from *in vivo* expression of ODC1 and NOS in conceptuses and endometria also indicate that Arg conversion into polyamines and NO will affect conceptus development during the peri-implantation period (325). Therefore, it was hypothesized that Arg increases production of NO and polyamines by oTr cells, and that these products increase mTOR cell signaling for cell proliferation. To our knowledge, results of the present study are the first to indicate that increased biosynthesis of polyamines and NO is induced by Arg to enhance cell signaling pathways that stimulate proliferation in ovine trophoblast cells. Further, results of the present study provide evidence that NO donors (SNAP and DETA) and a polyamine precursor (putrescine) increase proliferation of oTr cells by up to 3.8- and 6.7- fold. This effect depends on both NO and polyamine biosynthesis, since effects of Arg were reduced by 28.8% and 58.4%, respectively by a NOS inhibitor (L-NAME) and an arginase inhibitor (nor-NOHA). It is important to note, however, that in other cell types, NO may exert antiproliferative effects (357, 358) as generation of NO and polyamines compete for Arg as the common biosynthetic substrate for NOS and ODC.

Cell growth and hypertrophy occurs when protein synthesis exceeds the rate of protein degradation (287). In the ewe, trophoblast elongation is initiated around gestational Day 11 and occurs before implantation as conceptuses dramatically transition from spherical to tubular and elongated filamentous forms (23). During the process of elongation, size of the ovine conceptus increases more than 500-fold (32) due to an increase cell number and increased rates of protein synthesis (359). Results of the present study indicated that oTr cells respond to Arg with increases in protein synthesis and a parallel increase in cellular proliferation. Arg also stimulated the synthesis and release of IFNT, which is the most abundantly synthesized and secreted protein from mononuclear trophoblast cells of ruminant conceptuses (345). This result is another indicator of the stimulatory effect of Arg on protein synthesis in oTr cells. Similarly, Arg increases net protein synthesis in skeletal muscle of pigs, rats, and mice (194, 360-362), as well as intestinal cells (340). Indeed, increased availability of Arg in the ovine uterine lumen (95) is correlated with the increase in endometrial expression of transporters for Arg such as SLC7A2 which is induced at the onset of blastocyst elongation by progesterone and further increased by trophoblast-derived IFNT (297). In addition, it has been reported that mTOR inhibits protein degradation (363), which is consistent with the effect of Arg on oTr cells in the present study. This effect of Arg can result from the activation of mTOR signaling to increase phosphorylation of mTOR, RPS6K and 4EBP1 (Fig. 6.5).

Activated mTOR phosphorylates RPS6K and 4EBP1 *in vivo* and *in vitro* (98). There is increasing evidence that Arg can activate mTOR and other kinase-mediated signaling pathways to increase protein synthesis by increasing total and phosphorylated RPS6K (364), and also increase cell migration (304, 305). Consistent with these reports, results of the present study indicate that addition of Arg to culture medium activated the mTOR signaling pathway partly through phosphorylation of RPS6K and 4EBP1, stimulation of proliferation of oTr cells through a mechanism involving NO and polyamines and stimulation of migration of oTr cells through a NO-dependent mechanism.

Besides regulation of translation, mTOR has been reported to shuttle between the nucleus and cytoplasm for regulation of ribosome biogenesis, macroautophagy or

transcription (100, 365-367). We previously demonstrated that the abundance of p-mTOR and p-RPS6K increased in nuclei of conceptus trophoctoderm and endoderm (111, 268). Therefore it was important to investigate the intracellular localization of 4EBP1 in the present study. Results indicated that p-4EBP1 is present in the nuclei of Arg-treated oTr cells and that this effect was abrogated by rapamycin. The presence of p-4EBP1 in both the cytoplasm and in the nucleus has been observed in our laboratory and by others (368-370). While the significance of 4EBP1 in the nucleus is not known, its target protein, eIF4E, has been reported to function as a nuclear regulator of the export of a wide variety of RNAs involved in proliferation and growth of cells (237). In the future we will investigate the functional significance of nuclear translocation of p-4EBP1.

In conclusion, Arg added to culture medium at physiological concentrations stimulated proliferation and migration in oTr cells through mechanisms mediated by NO and polyamine synthesis, increased protein synthesis and reduced protein degradation. These effects of Arg, mediated via the mTOR cell signaling pathway, provide important insights for beneficial effects of dietary supplementation with Arg to improve fetal-placental growth and development, as well as survival and growth of offspring during the neonatal period.

CHAPTER VII

DIFFERENTIAL EFFECTS OF ARGININE, LEUCINE, GLUTAMINE AND GLUCOSE ON EXPRESSION OF INTERFERON TAU, ORNITHINE DECARBOXYLASE AND NITRIC OXIDE SYNTHASE BY EXPLANT CULTURES OF OVINE CONCEPTUSES FROM DAY 16 OF PREGNANCY

Introduction

In sheep, blastocysts undergo morphological transitions from spherical to tubular and then elongated filamentous forms that develop into conceptuses (embryo/fetus and its associated extra-embryonic membranes) during the peri-implantation period of pregnancy (4, 41). During the peri-implantation period, elongation of ovine conceptuses is dependent on uterine secretions, referred to as histotroph, which is a complex mixture that includes growth factors, hormones, cytokines, ions, lipids, glucose and amino acids (1, 3). Studies of the uterine gland knockout ewe (UGKO) indicated that secretions from the GE are required for conceptus survival and elongation of trophoctoderm as conceptuses were unable to elongate in the absence of uterine glands (5, 6, 63, 68). Among the components of histotroph are nutrients required for energy, metabolism and cell signaling. Growing evidence suggests that the amino acids and glucose have a role as activators of protein synthesis in cells and tissues; however, only recently have their roles in modulating intracellular signal transduction pathways been described (371).

Amino acids are not only cell signaling molecules (119, 205, 287), but also major energy sources for fetal-placental growth (201, 202, 215, 292), regulators of gene expression and stimulators of the protein phosphorylation cascade. Additionally, amino acids are key molecules in synthesis of hormones (290, 291) and nitrogenous substances that each has enormous biological importance (287-289). Of particular interests is arginine (Arg) which is a common substrate for production of NO (nitric oxide) and polyamines via NO synthase (NOS) and ornithine decarboxylase (ODC1) (372). After implantation, NO plays important roles to regulate placental angiogenesis (293) and placental-fetal blood flows to increase the transfer of nutrients and oxygen from mother to fetus (294, 295). In mouse trophoblast, NO generated by iNOS and eNOS stimulate trophoctoderm motility by inducing cGMP and modifying the ECM. In ovine

placentomes, increases in NOS2 (inducible) and NOS3 (endothelial) activities (333) stimulate placental NO synthesis, which is closely associated with increases in placental vascular growth and utero-placental blood flow in ewes during pregnancy (350). In NOS activity, guanosine triphosphate cyclohydrolase (GCH1) is a rate-limiting enzyme in the production of tetrahydrobiopterin (BH4), which is an essential cofactor for all isoforms of NOS (373). The polyamines also are essential for placental development and mammalian embryogenesis (296). Indeed, polyamines are required for DNA and protein synthesis and, therefore, cell proliferation and differentiation (Flynn et al., 2002). Regulation of mTOR signaling by leucine (Leu) has been studied extensively in rodents (374, 375) and in humans (376). Further, Arg and Leu are required for trophoblast outgrowth and blastocyst motility in mice (22, 96). Glucose and glutamine (Gln) are major nutrients for conceptuses as they enhance trophoblast cell proliferation by activating the glutamine-fructose-6-phosphate-amidotransferase (GFAT)-mediated target of rapamycin (mTOR, formerly FRAP1) cell signaling pathway (21).

The nutrient-induced stimulation of protein synthesis is mediated by mTOR signaling (377) that integrates amino acid availability, cellular energy status, and endocrine signals to regulate protein synthesis through control of the phosphorylation status and, therefore, state of activation of eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1) and ribosomal protein S6 kinase-1 (RPS6K1). Phosphorylation of repressor protein, 4E-BP1, results in its dissociation from eIF4E, allowing it to bind to eIF4G to form complexes that mediate binding of mRNA to the 40S ribosomal complex in the initiation of mRNA translation (255). Phosphorylation of RPS6K1 leads to activation of several components of the protein translation apparatus (378).

The purpose of the present study was to determine the differential effects of Arg, Leu, Gln and glucose on expression of total and phosphorylated forms of protein in the mTOR cell signaling pathway as well as translation of mRNAs for key molecules associated with conceptus development such as NOSs, ODC, GCH1 and IFNT. To investigate the direct effects of nutrients on protein synthesis, the experiments were conducted by using explant cultures of conceptuses immediately after recovery from uteri of Day 16 pregnant ewes.

Materials and Methods

Preparation of ovine conceptuses

Using procedures described previously (342, 343), conceptuses from Day 16 pregnant ewes (coincident with maximal production of IFNT by the conceptuses) were recovered by flushing each uterine horn with 20 ml customized medium (90) containing approximately one-tenth the amount of each amino acid and glucose present in fully supplemented culture medium. The conceptuses were starved for 6 h in customized medium (i.e., lacking the select nutrient of interest). For example, Arg-free medium was used initially for the Arg treatment group. Then conceptuses were cultured in 0.2 mM Arg, 0.2 mM Leu, 0.5 mM Gln or 4 mM glucose for 18 h at 37°C on a rocking platform under an atmosphere of 50% O₂, 45% N₂, and 5% CO₂. After incubation, the conceptuses were homogenized, the homogenate assayed for protein and then stored at -80°C until analyzed.

RNA isolation

Total cellular RNA was isolated from cultured conceptuses using TRIzol reagent (Life Technologies, Inc.-BRL, Bethesda, MD) according to manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

Cloning of partial cDNAs for ovine conceptus mTOR, RPS6K, RPS6, 4EBP1, NOSs, GCH1, IFNT and ODC1

Partial cDNAs for ovine *mTOR*, *RPS6K*, *RPS6*, *4EBP1*, *NOSs*, *GCH1*, *IFNT* and *ODC1* mRNAs were amplified by RT-PCR using total RNA from conceptuses and previously described primers (111, 158, 268, 325) and methods as described previously (158). The partial cDNAs of the correct predicted size were cloned into pCRII using a T/A Cloning Kit (Invitrogen Corp., Carlsbad, CA), and the sequence of each was verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (PerkinElmer Applied Biosystems, Foster City, CA).

Western blot analysis

Whole conceptus extracts and immunoblot assays were prepared and performed as described previously (234, 268) . To harvest total cellular protein for western blot analyses, conceptuses were rinsed with cold PBS and lysed by homogenization in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na₃VO₄, 0.2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 30 mM Na₄P₂O₇, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 30 min at 4°C. Cell lysates were clarified by centrifugation (16,000 x g, 15 min, 4°C). The protein content was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured, separated using SDS-PAGE, transferred to nitrocellulose and western blotting performed as described previously (268) using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and X-OMAT AR X-ray film (Kodak, Rochester, NY) according to manufacturer's recommendations. All antibodies for mTOR signaling molecules including RPS6K, RPS6 and 4EBP1 were purchased from Cell Signaling Technology (Danvers, MA). Immunoreactive proteins were detected by using rabbit anti-mouse polyclonal phospho-mTOR IgG (Catalog numbers 2971) at a dilution of 1:1,000 and mTOR IgG (Catalog numbers 2972) at a dilution of 1:2,000. Following separation of proteins on 10% SDS-PAGE gels the following antisera were used at the noted dilutions: rabbit anti-human polyclonal phospho-RPS6K IgG (Thr421/Ser424) (Catalog number 9204; dilution of 1:1,000) RPS6K IgG (Catalog number 9202; dilution of 1:2,000); , rabbit anti-human polyclonal phospho-4EBP1 IgG (Thr37/46) (Catalog number 2855; 1:1,000 dilution); 4EBP1 IgG (Catalog numbers 9452; 1:1,000 dilution); rabbit anti-human polyclonal phospho-RPS6 IgG (Catalog number 2215; 1:1,000 dilution); RPS6 IgG (Catalog numbers 2972; 1:2,000 dilution); mouse anti-NOS3 polyclonal IgG (catalog no. 610297; BD Transduction Laboratories. 1 µg/ml), purified rabbit anti-rat GCH1 polyclonal IgG (produced by Drs. C. J. Meininger and G. Wu, Texas A&M University, College Station, TX; catalog no. 89980, Thermo Scientific, 2 µg/ml) and purified rabbit anti-ODC1 polyclonal IgG (catalog no. HPA001536; Atlas Antibodies AB, Stockholm, Sweden, 0.5 µg/ml) at final concentrations of 2 µg/ml, 2 µg/ml, 1 µg/ml, 2 µg/ml, 2 µg/ml, and 0.5 µg/ml, respectively (325). As a loading control, western blotting with

mouse anti-alpha tubulin (TUBA) IgG (Catalog number; T5168; Sigma, St. Louis, MO) was performed. Multiple exposures of each western blot were conducted to ensure linearity of chemiluminescent signals. Western blots were quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA).

Statistical analyses

All quantitative data were subjected to least squares analyses of variance (301) using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Western blot data were corrected for differences in sample loading using the TUBA data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P-value ≤ 0.05 was considered significant. Data are presented as least-square means (LSM) with standard errors (SE).

Results

Regulation of mTOR, RPS6K, RPS6 and 4EBP1 mRNAs by arginine, leucine, glutamine and glucose

To determine whether Arg, Leu, Gln and glucose increase the expression of genes of the mTOR pathway associated with conceptus development, we performed RT-PCR using specific primers for mTOR, RPS6K, RPS6 and 4EBP1 mRNAs and found constitutive expression of these genes in untreated and treated conceptuses, but there was no effect of addition of Arg, Leu, Gln or glucose on expression of any of those mRNAs (Fig. 7.1).

Regulation of NOSs, ODC, GCH1 and IFNT mRNAs by arginine, leucine, glutamine and glucose

The expression of mRNAs for *NOS2* (inducible), *NOS3* (endothelial), *ODC*, *GCH1* and *IFNT* was evaluated by RT-PCR (Fig.7.2). Effects of Arg, Leu, Gln and glucose on expression of these genes in explant cultures of ovine conceptuses were not detected.

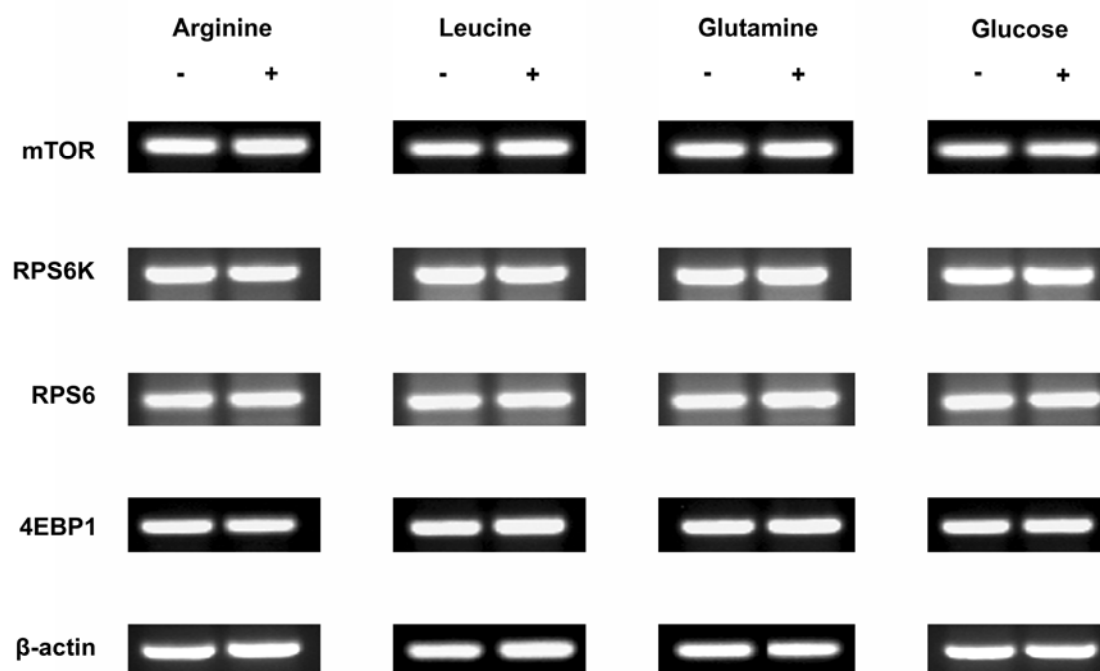


Fig. 7.1. Expression of mTOR, RPS6K, 4E-BP and RPS6 transcripts in response to arginine (Arg), leucine (Leu), glutamine (Gln) and glucose (Gluc) in ovine conceptuses evaluated by RT-PCR. Conceptuses were isolated from Day 16 pregnant ewes, washed with customized medium, starved in selected nutrient free medium (e.g., Arg free medium for Arg treatment group) and then treated with either 0.2 mM Arg, 0.2 mM Leu, 0.5 mM Gln or 4 mM glucose. Following incubation, total cellular RNA was isolated, the quantity and quality of total RNA were determined, and control gene β -actin were run on 1% agarose gels.

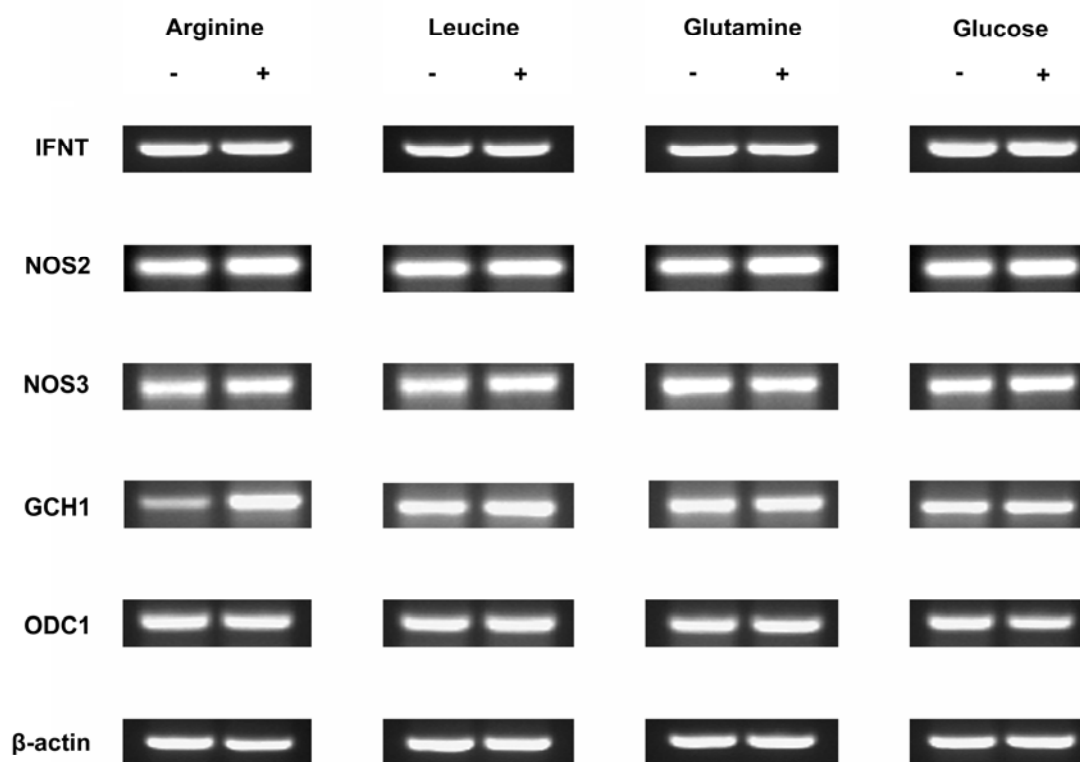


Fig. 7.2. Expression of NOS2, NOS3, ODC1, GCH1 and IFNT transcripts in response to arginine (Arg), leucine (Leu), glutamine (Gln) and glucose (Gluc) in ovine conceptuses evaluated by RT-PCR. Conceptuses were isolated from Day 16 pregnant ewes, washed with customized medium, starved in selected nutrient free medium (e.g., Arg free medium for Arg treatment group) and then treated with either 0.2 mM Arg, 0.2 mM Leu, 0.5 mM Gln or 4 mM glucose. Following incubation, total cellular RNA was isolated, the quantity and quality of total RNA were determined, and control gene β -actin were run on 1% agarose gel.

However, Arg treated conceptuses expressed increased amounts of *GCHI* mRNA compared to untreated conceptuses .

Effects of arginine, leucine, glutamine and glucose on mTOR, RPS6K, RPS6 and 4EBP1 proteins

The effects of Arg, Leu, Gln and glucose on the relative protein levels for both total and phosphorylated forms of mTOR, RPS6K, 4EBP1 and RPS6 were determined by western blotting. Compared with the control explants cultures of conceptuses, Arg increased levels of total mTOR and RPS6K by 2.4- and 2.3- ($P < 0.01$) fold while Leu increased mTOR and RPS6K by 2.0- and 1.9- ($P < 0.01$) fold. The effects of Arg and Leu on phosphorylation of mTOR and RPS6K were similarly significant. The abundance of total mTOR and RPS6K proteins was increased 2.4- and 2.4- ($P < 0.01$) fold by glucose as was the abundance of phosphorylated mTOR and RPS6K ($P < 0.01$). In conceptuses treated with Gln, total mTOR, RPS6K and p-RPS6K were increased by 1.8-, 1.9- and 2.0- ($P < 0.01$) fold, but the increase in p-mTOR was not significant (Fig. 7.3A).

Western blot analyses of conceptus extracts with antibodies to total RPS6 and 4EBP1 proteins indicated that Arg increased total RPS6 and 4EBP1 by 3.1- and 3.2- ($P < 0.05$) fold, while Leu increased their abundance by 2.0- and 2.2- ($P < 0.01$) fold. The addition of Arg to culture medium increased protein levels of phosphorylated forms of RPS6 and 4EBP1 by 3.2- and 4.9- ($P < 0.05$) fold. Leu increased the levels of phosphorylated form of RPS6 and 4EBP1 by 2.1- and 2.3- fold ($P < 0.01$). The levels of total RPS6 and 4EBP1 proteins were increased by 2.2- and 2.3- ($P < 0.01$) fold by Gln and the effects of Gln on phosphorylated RPS6 and 4EBP1 were similar. Treatment of conceptuses with glucose increased total RPS6 and 4EBP1 by 3.3- ($P < 0.05$) and 2.3- ($P < 0.01$) fold, and phosphorylated RPS6 and 4EBP by 2.9- ($P < 0.01$) and 4.9- ($P < 0.05$) fold (Fig. 7.3B).

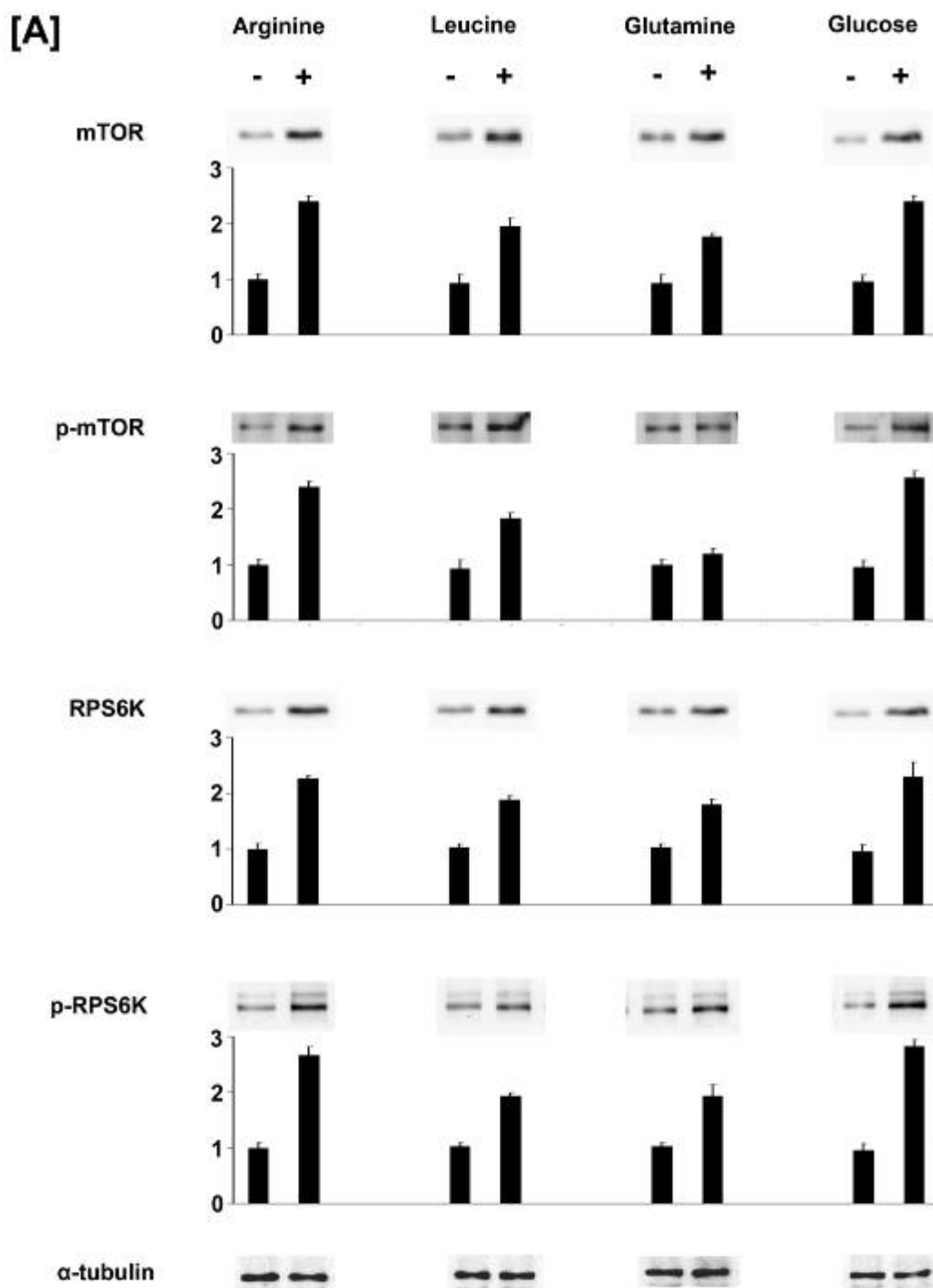


Fig. 7.3. Relative increases in total and phosphorylated mTOR, RPS6K, 4E-BP and RPS6 proteins by arginine (Arg), leucine (Leu), glutamine (Gln) and glucose (Gluc). Conceptuses were isolated from Day 16 pregnant ewes, washed with customized medium, starved in selected nutrient free medium (e.g., Arg free medium for Arg treatment group) and then treated with either 0.2 mM Arg, 0.2 mM Leu, 0.5 mM Gln or 4 mM glucose. Following incubation, total proteins were extracted, assayed and subjected to SDS-PAGE gel for analysis. Western blotting images are of representative results.

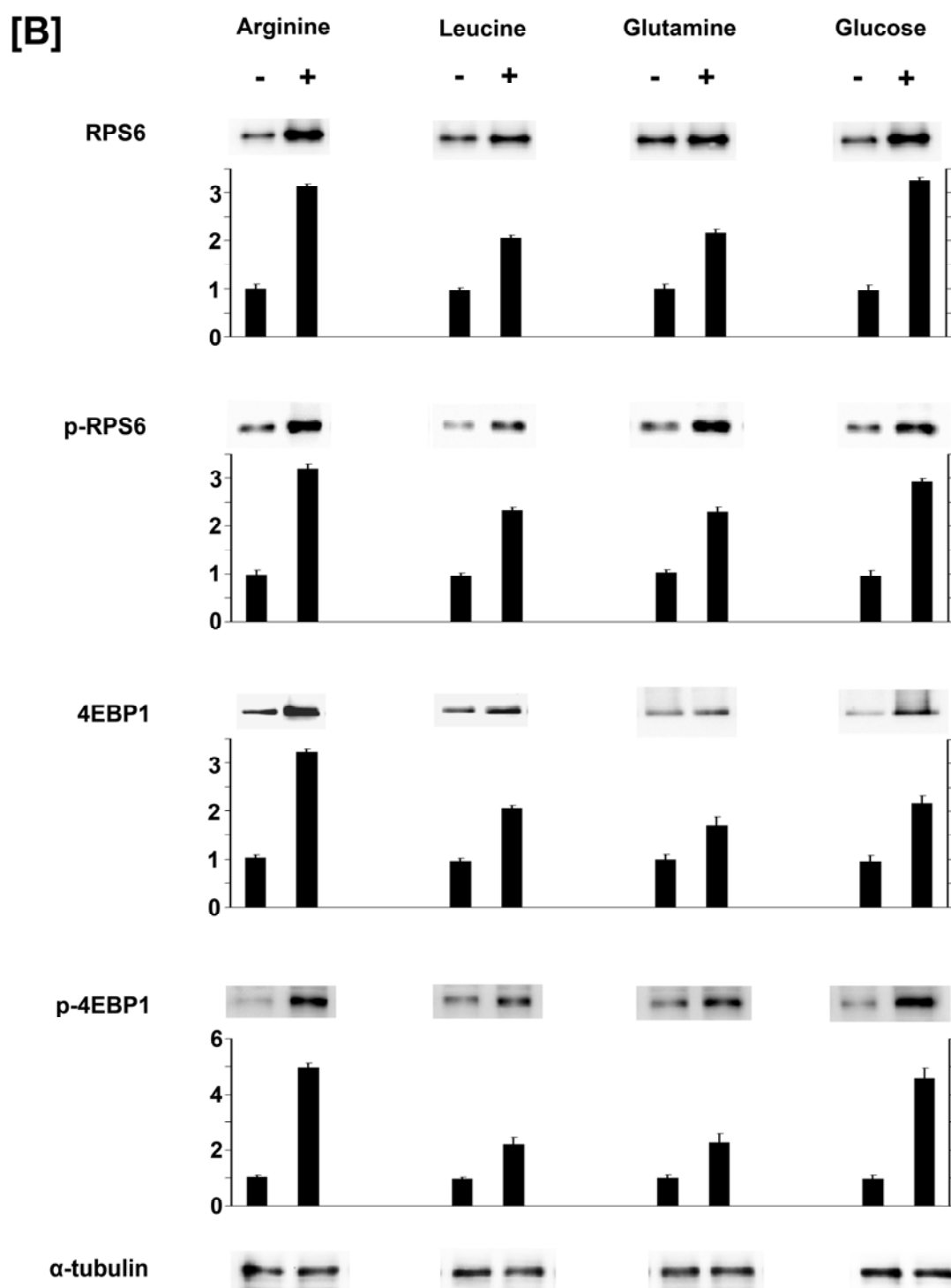


Fig. 7.3 continued.

Effects of arginine, leucine, glutamine and glucose on NOS2, NOS3, ODC1, GCH1 and IFNT proteins

To investigate the effects of Arg, Leu, Gln and glucose on protein synthesis, conceptus proteins were evaluated using SDS-PAGE and immunoblotting with antibodies against NOS2, NOS3, ODC1 and GCH1 proteins (Fig. 7.4). In response to Leu, Gln and glucose, there was no effect on abundance of IFNT. However, Arg stimulated IFNT synthesis by 2.3- ($P < 0.01$) fold compared with controls. In addition, Arg stimulated synthesis of ODC1, NOS2 and NOS3 by 2.0-, 2.1- and 2.3 fold ($P < 0.01$). For conceptuses treated with Leu and Gln, expression of ODC1 and NOS2 were not increased, but abundance of NOS3 was increased 2.0-fold ($P < 0.01$) by Gln. GCH1 abundance was increased by Arg and glucose, 2.2- and 2.1- ($P < 0.01$) fold, respectively, whereas Leu and Gln had no effect on abundance of GCH1.

Discussion

The availability of nutrients in the uterine lumen determine conceptus survival and, growth and development during the critical peri-implantation period of pregnancy in mammals (1, 63). Available evidence suggests that nutritional deficiencies during early pregnancy lead to a poorer outcome of pregnancy (1, 21, 63, 213), as well as have an adverse impact on postnatal growth and health (119). Mammalian target of rapamycin (mTOR) responds to alterations in amino acid and glucose availability and controls transcription and translation of proteins associated to cell proliferation and growth (22, Wen, 2005 #1237, 374). The RNAs and proteins in the mTOR pathway are expressed in ovine conceptus endoderm and trophoblast (111); however, the nutritional factors affecting protein translation in the ovine conceptus, and the molecular mechanisms mediating their effects, are not known. The present study was to assess the role of mTOR cell signaling pathway in the regulation of synthesis of IFNT, NOS, ODC and GCH1 proteins by select nutrients.

In both yeast and mammals, mTOR regulates transcription, as well as rRNA processing and mRNA translation. Inhibition of the mTOR pathway with rapamycin or by nutrient starvation results in a down-regulation of transcription of mRNAs (379, 380), tRNA, and rRNA (381, 382). Therefore, the present study first determined whether Arg,

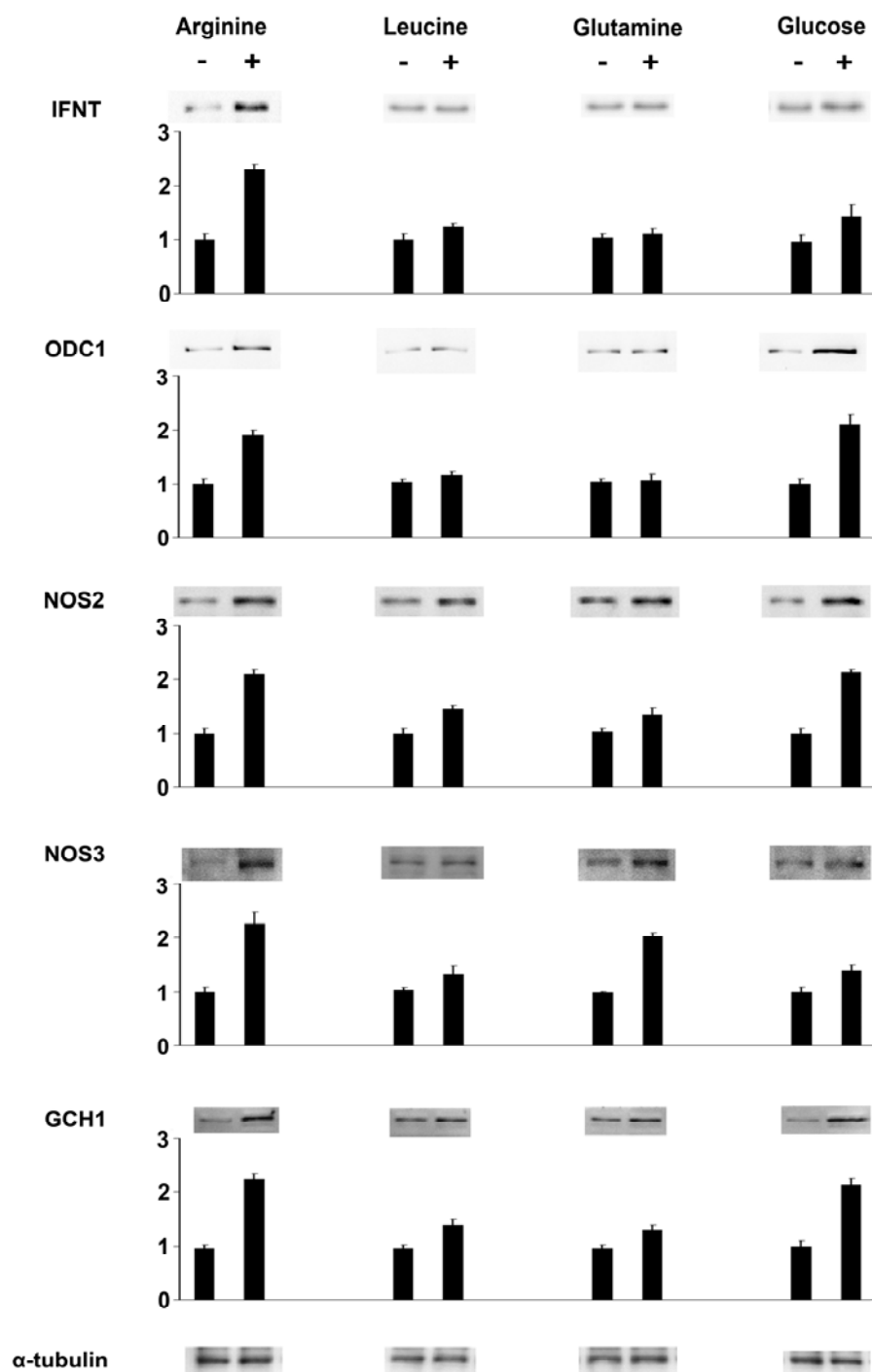


Fig. 7.4. Relative increases in NOS2, NOS3, ODC1, GCH1 and IFNT proteins in response to arginine (Arg), leucine (Leu), glutamine (Gln) and glucose (Gluc). Conceptuses were isolated from Day 16 pregnant ewes, washed with customized medium, starved in selected nutrient free medium (e.g., Arg free medium for Arg treatment group) and then treated with either 0.2 mM Arg, 0.2 mM Leu, 0.5 mM Gln or 4 mM glucose. Following incubation, total proteins were extracted, assayed and subjected to SDS-PAGE gel for analysis. Western blotting images are of representative results.

Leu, Gln and glucose induced gene expression *in vitro* in explant cultures of ovine conceptuses from Day 16 of pregnancy. Results from present study showed that these amino acids and glucose did not induce gene transcription, except that Arg increased expression of *GCH1* mRNA. Although this increase of *GCH1* mRNA was not confirmed quantitatively, it is likely that Arg affects GCH1 gene transcription since *GCH1* mRNA is very abundant in ovine conceptuses during the peri-implantation period of pregnancy (325). GCH1 is the key enzyme for synthesis of BH4 (tetrahydrobiopterin), an essential cofactor for NOS activity (383), so increased GCH1 expression would stimulate NO synthesis through increased activity of isoforms of NOS. This hypothesis could explain the “arginine paradox” which has been used to refer to the dependence of cellular NO production on exogenous Arg administration despite the theoretical saturation of NOS enzymes with intracellular Arg. To identify this mechanism, further investigation is needed to determine if Arg-stimulates transcription of *GCH1* gene(s) and whether GCH1 expression supports cell growth by increasing NOS activity.

We next examined expression of proteins by Arg, Leu, Gln and glucose and found that that these select nutrients increased phosphorylation of mTOR and that this change was associated with increased abundance of key proteins in the mTOR pathway . This effect may also reflect a decrease in the amount of degradation of proteins (194, 361). Additionally, Arg, Leu, and Gln stimulated the phosphorylation of RPS6K, RPS6 and 4EBP1 which is consistent with reports of increases in protein synthesis and translational efficiency in cells (304, 340, 384, 385). Results of the present study extend the original observation that Arg and Leu stimulate phosphorylation of proteins of the mTOR signaling pathway in ovine conceptuses (unpublished data). Although we previously showed that addition Gln to culture medium failed to change the levels of phosphorylated proteins in the mTOR cell signaling pathway in trophoblast cells, Gln did increase protein synthesis in explants cultures of Day 16 ovine conceptuses in the present study. These differential effects of Gln on ovine trophectoderm cells versus the explants cultures of ovine conceptus trophoblast may indicate differential effects on cell type, cell activity and presence of extra-embryonic endoderm which has an intense level of metabolism and energy consumption that may change uptake of Gln and its possible use as an energy source to compliment that of glucose metabolism (386). This finding of

differential effects of Arg and Leu versus Gln may be particularly noteworthy because mTOR represents a bifurcation point for distal parallel signaling pathways, leading to the phosphorylation of 4E-BP1 and RPS6K, and it also appears to represent a signal integration center for the stimulatory effects of growth factors and nutrients including glucose on cellular functions (99, 387, 388).

Glucose is the primary energy source for embryos/conceptuses and its availability for oxidation and glycolysis increases between Days 13 and 19 of pregnancy (389, 390), which is coincident with the rapid elongation period to form filamentous conceptuses (63). Metabolism of glucose through glycolysis and the pentose cycle generates ATP and NADPH required for production of NO for oxidative defense and ribose-5-phosphate for nucleotide synthesis. Glucose stimulates RPS6K via the mTOR kinase, which functions as an ATP sensor (391) and also is a mitogen for trophoblast cell growth and proliferation through the GFAT-mediated mTOR cell signaling pathway (21).

In the present study, glucose alone increased mTOR phosphorylation and its downstream effectors, RPS6K and 4EBP1. These results are consistent with those reported for muscle cells (392), in which glucose activates phosphorylation of RPS6K. Similarly, the administration of glucose increases mucosal and muscle protein synthesis (393, 394) and such increases result because of the enhanced rate of protein initiation rather than the elongation step in translation. The accelerated rate of protein synthesis in response to glucose is concomitant with increases in amounts of inactive 4EBP1 and increased phosphorylation and activation of RPS6K and RPS6 (392).

Further, we examined the ability of Arg, Leu, Gln and glucose to increase abundance of NOS2, NOS3, ODC, GCH1 and IFNT proteins in explants cultures of ovine conceptuses because molecules that stimulate mTOR activity in trophectoderm can stimulate translation of mRNAs critical to conceptus development, including *ODC* and *NOS* (22, 114). Results of the present study provide the initial evidence that amino acids and glucose affect expression of NOS3, GCH1, and ODC1 on ovine conceptuses and that the effects are likely mediated through increased phosphorylation and activation S6K1 and 4EBP1. Indeed, expression of NOSs and ODC1 proteins is essential for conceptus development since Arg is the substrate for producing NO and polyamines via arginase and ODC1, as well as stimulating intracellular signaling, cell proliferation, and

protein synthesis (197, 395). It is also important to note that expression of GCH1 protein is essential for BH4 production and NOS activity. Previous work indicated that GCH1 activity is correlated positively with NO production in ovine placentae and uterine endometria between Days 30 and 140 of pregnancy (333), an effect greatly influenced by changes in BH4 availability (383). Available evidence indicates that expression of GCH1 protein and mRNA may be differently regulated at transcriptional and translational levels (383). An important finding in the present study was that the Arg is most effective in stimulating protein synthesis, mTOR signaling, IFNT synthesis and abundance of essential components of the mTOR pathway, as well as a potent stimulator of cell proliferation and cell migration.

In conclusion, Arg, Leu, Gln and glucose differentially increase NOS2, NOS3, ODC1, GCH1 and IFNT by explants cultures of Day 16 ovine conceptuses. This effect was associated with increased abundance of phosphorylated and activated mTOR, RPS6K, and RPS6, as well as inactivation of 4EBP1. These results provide important evidence that availability of select nutrients can increase expression of cell signaling molecules and affect conceptus growth, development, and survival through stimulation of protein synthesis during the peri-implantation period of pregnancy.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Summary

Histotroph, secretions from uterine epithelia and selective transport of molecules from maternal serum, is required for conceptus elongation and development as well as successful pregnancy. The results from UGKO ewes revealed a requirement for secretions from LE and GE to support conceptus development beyond Day 14 (5, 6). Despite the importance of secreted factors from the endometrium, little is known about functions and mechanism of histotroph on conceptus. The present research focused on elucidating the mechanisms whereby components of histotroph including IGF2 (growth factor), SPP1 (cell matrix protein), amino acids and glucose (nutrients) affect the mTOR signaling pathway, and to determine the functional roles of these selected components of histotroph using ovine trophectoderm (oTr) cells and explants cultures of Day 16 ovine conceptuses to gain insight into how they may affect survival and development of ovine conceptuses.

IGF2, a potent stimulator of cellular proliferation, differentiation, and development, regulates uterine functions and conceptus growth in several species; however, the expression pattern of *IGF2* in peri-implantation ovine conceptuses and its mechanism of action was not well defined. Therefore, experiments outlined in Chapter III determined temporal and cell specific changes in expression of IGF2 in uterine endometria of cyclic (Days 10-16) and early pregnant (Days 10-20) ewes, as well as in peri-implantation conceptuses (Days 13-20 of gestation) from ewes. *In situ* hybridization analyses found that *IGF2* mRNA was most abundant in the caruncular endometrial stroma of both cyclic and pregnant ewes. In the intercaruncular endometrium, *IGF2* mRNA transitioned from stroma to luminal epithelium (LE) between Days 14 and 20 of pregnancy. *IGF2* mRNA was present in all cells of the conceptus, but was particularly abundant in the primitive endoderm and yolk sac. To understand the mechanisms by which IGF2 plays a critical role in conceptus survival and development, we investigated the cell signaling pathway and its functional consequence on ovine trophectoderm cells. In oTr cells isolated from Day 15 conceptuses, IGF2 increased the

abundance of p-PDK1, p-AKT1, p-GSK3B, mTOR/FRAP1, and p-RPS6K protein within 15 min, and the increase was maintained for 90 min. IGF2 also elicited a rapid increase in p-ERK1/2 and p-P38 MAPK proteins that was maximal at 15 or 30 min post-treatment. Moreover, IGF2 increased migration of oTr cells. Collectively, these results support the hypothesis that IGF2 coordinately activates multiple cell signaling pathways critical to survival, growth, and differentiation of the ovine conceptus during early pregnancy.

Attachment and migration of trophectoderm cells, hallmarks of blastocyst implantation in mammals, are unique uterine events. Secreted phosphoprotein 1 (SPP1) in the uterus binds integrins on conceptus trophectoderm and uterine LE, affecting cell-cell and cell-matrix interactions. The signal transduction pathways activated by SPP1 and integrins in conceptuses have not been elucidated. Results of this study demonstrated that SPP1 binds $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins to induce focal adhesion assembly, a prerequisite for adhesion and migration of trophectoderm, through activation of: 1) P70S6K via crosstalk between FRAP1/MTOR and MAPK pathways; 2) MTOR, PI3K, ERK1/2 and P38 MAPK signaling to stimulate oTr cell migration; and 3) focal adhesion assembly and myosin II motor activity to induce migration of oTr cells. These cell signaling pathways, acting in concert, mediate adhesion, migration and cytoskeletal remodeling of oTr cells essential for expansion and elongation of conceptuses and attachment to uterine LE for implantation.

Histotroph is required for survival and development of ovine conceptuses (embryo and extra-embryonic membranes). Results from our laboratory indicate that arginine (Arg), leucine (Leu), glutamine (Gln) and glucose increase in the uterine lumen between Days 10 and 15 of pregnancy, coincident with increases in expression of amino acid and glucose transporters by uterine epithelia, as well as trophectoderm and yolk sac of conceptuses. Therefore, we hypothesized that Arg, Leu, Gln and glucose have differential effects on hypertrophy, hyperplasia and differentiated functions of cells critical to ovine conceptus development. Primary oTr cells isolated from Day 15 conceptuses were serum-starved for 24h in a customized medium, deprived of select nutrients and then treated with either Arg, Leu, Gln or glucose. Western blot analyses of whole oTr cell extracts showed that Arg, Leu and glucose, but not Gln, increased phosphorylated AKT1 (pAKT1) 2.8-, 2.5- and 1.8- fold, respectively, within 15 min and

the increase was maintained to 60 min. Arg, Leu and glucose also stimulated 4.2-, 4.7-, and 2.3-fold increases in phosphorylated RPS6K within 15 min, as well as increases in pRPS6 protein between 0 and 30 min post-treatment that were sustained to 60 min. When oTr cells were treated with Arg, phosphorylated RPS6K proteins increased in nuclei, but remained low in nuclei of cells treated with Leu and glucose. Immunocytochemical analyses also revealed abundant amounts of phosphorylated RPS6 proteins in the cytoplasm of oTr cells treated with Arg, Leu and glucose. Further, Arg and Leu increased ($P<0.001$) proliferation and migration of oTr cells. Collectively, these results indicate that Arg, Leu and glucose, but not Gln, in histotroph coordinately activate AKT1-MTOR and RPS6K-RPS6 signaling pathways to stimulate hypertrophy, hyperplasia, and/or migration of conceptus trophoctoderm.

During the peri-implantation period in sheep, L-arginine (Arg) in the uterine lumen is an essential substrate for synthesis of nitric oxide and polyamines required for survival and development of ovine conceptuses. The hypothesis that Arg stimulates hypertrophy, hyperplasia and differentiation of ovine conceptus trophoctoderm is supported by results from our laboratory indicating significant increases in Arg availability in the uterine lumen between Days 10 and 15 of pregnancy, expression of cationic amino acid transporters by uterine epithelia and conceptuses, and abundant ornithine decarboxylase (ODC) and nitric oxide synthase (NOS) expression by oTr cells. It was also found that Arg activates the mTOR/FRAP1 and the RPS6K cell signaling pathway. Therefore, this study examined effects of Arg on signal transduction, and the effects of Arg and the NO donors, SNAP and DETA, or putrescine (precursor for spermidine and spermine) on oTr cell proliferation. Further, the inhibition of these effects by L-NAME (an inhibitor of NOS) and Nor-NOHA (an inhibitor of arginase) was assessed. Arg treatment increased relative protein levels for p-MTOR, p-RPS6K and p-4EBP1. Consistent with activation of these signaling pathways, Arg increased protein synthesis and reduced protein degradation in oTr cells. Both NO and polyamines enhanced cell proliferation in a dose-dependent manner. The effects of Arg were partially inhibited by both L-NAME and Nor-NOHA. These results indicate that Arg enhances production of polyamines and NO and activates the MTOR/FRAP1-RPS6K-RPS6 signaling pathway to stimulate proliferation of oTr.

Nutrients are a primary requirement to stimulate protein synthesis, and development of ovine conceptuses. It was shown Arg, Leu, and glucose stimulate protein synthesis through the phosphorylation of mTOR signaling molecules, thereby leading to an increase in cell proliferation in trophoblast cells. However, Gln did not elicit those responses. Therefore, one study aimed to clarify whether Arg, Leu, Gln and glucose exposure influenced gene expression and protein synthesis in explants cultures of conceptuses from Day 16 of pregnancy. The ovine conceptuses isolated from Day 16 pregnant ewes were deprived of select nutrients and then cultured with either Arg, Leu, Gln or glucose. After incubation for 18 h, the levels of mRNA and protein for mTOR, RPS6K, 4EBP1 and RPS6 were analyzed by using RT-PCR and immunoblot analysis. Further, the expression of mRNAs and proteins for NOS, ODC1, GCH1 and IFNT were evaluated in conceptus tissue. Expression of mTOR, RPS6K, 4EBP1 and RPS6 mRNAs was not different between untreated and treated conceptuses. ODC1, NOS, and IFNT mRNAs were detectable, but there was no significant difference among the Arg, Leu, Gln and glucose treated and control group. Interestingly, GCH1 mRNA levels increased in Arg-treated explants cultures of ovine conceptuses. Western blot analyses of conceptus extracts showed that Arg, Leu, Gln and glucose did increase phosphorylation of mTOR, RPS6K, 4EBP1 and RPS6, and that these increases were reflected by increased levels of total protein. Protein analyses also revealed that Arg and glucose were most effective in increasing the amounts of ODC1, NOS2 and GCH1 whereas Leu and Gln fail to increase synthesis of ODC1 and NOS proteins. Importantly, only conceptuses treated with Arg significantly increase IFNT synthesis. These findings suggest that Arg, Leu, Gln and glucose differentially regulate protein synthesis and that these effects are regulated through phosphorylation and activation of the mTOR cell signaling pathway.

Conclusions

In conclusion, results from the present studies provided the basis for a model whereby nutrients, SPP1 and IGF2 likely affect conceptus development. First, results of these studies provide strong evidence for SPP1-induced integrin-mediated force-generated migration and cell adhesion mechanisms in Tr cells utilizing mTOR, MAPK14 (p38), MAPK1/MAPK3 (Erk1/2), as well as a novel link between p38 and mTOR

signaling pathways (see Figs. 3.8 and 4.7A). Second, as shown in this model (see Fig. 4.7B), results of this study indicate that: 1) SPP1 serves as a key adhesive ECM protein that mediates Tr cell attachment, focal adhesion formation and reorganization of the cytoskeleton necessary for cell migration and attachment to uterine LE; 2) IGF2 and SPP1 activate PI3K and MAPK signaling pathways to affect Tr migration necessary for cellular and tissue reorganization during conceptus elongation (268); and 3) Arg induces proliferation of Tr cells required for conceptus development in sheep as has been reported for mice (22, 96). Collectively, SPP1, IGF2 and arginine activate mTOR signaling pathways mediated by AKT1, TSC1/2 and mTORC1 (cell proliferation and mRNA translation), as well as mTORC2 (cell migration, cell survival and cytoskeletal organization) in Tr cells to allow conceptuses to make the critical transition from spherical to tubular and filamentous forms that is a prerequisite for signaling pregnancy recognition, implantation and placentation required for successful pregnancy outcomes in all mammals.

REFERENCES

1. **Bazer FW** 1975 Uterine protein secretions: Relationship to development of the conceptus. *J Anim Sci* 41:1376-1382
2. **Bazer FW, Roberts RM, Thatcher WW** 1979 Actions of hormones on the uterus and effect on conceptus development. *J Anim Sci* 49 Suppl 2:35-45
3. **Roberts RM, Bazer FW** 1988 The functions of uterine secretions. *J Reprod Fertil* 82:875-892
4. **Spencer TE, Johnson GA, Bazer FW, Burghardt RC** 2004 Implantation mechanisms: insights from the sheep. *Reproduction* 128:657-668
5. **Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE** 2002 Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction* 124:289-300
6. **Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, Spencer TE** 2001 Endometrial glands are required for preimplantation conceptus elongation and survival. *Biol Reprod* 64:1608-1613
7. **Dennis PB, Pullen N, Kozma SC, Thomas G** 1996 The principal rapamycin-sensitive p70(s6k) phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases. *Mol Cell Biol* 16:6242-6251
8. **Thomas G, Hall MN** 1997 TOR signalling and control of cell growth. *Curr Opin Cell Biol* 9:782-787
9. **Gingras AC, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, Polakiewicz RD, Wyslouch-Cieszyńska A, Aebersold R, Sonenberg N** 2001 Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev* 15:2852-2864
10. **Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN** 2004 Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* 6:1122-1128
11. **Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, Um SH, Brown EJ, Cereghini S, Thomas G, Kozma SC** 2004 Disruption of the mouse

- mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. *Mol Cell Biol* 24:9508-9516
12. **Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofer F, Kiyama H, Yonezawa K, Yamanaka S** 2004 mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. *Mol Cell Biol* 24:6710-6718
 13. **Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM** 2006 Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev Cell* 11:859-871
 14. **Shiota C, Woo JT, Lindner J, Shelton KD, Magnuson MA** 2006 Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. *Dev Cell* 11:583-589
 15. **Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B** 2006 SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* 127:125-137
 16. **Bode BP** 2001 Recent molecular advances in mammalian glutamine transport. *J Nutr* 131:2475S-2485S; discussion 2486S-2477S
 17. **Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J** 1998 Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem* 273:14484-14494
 18. **Wang X, Campbell LE, Miller CM, Proud CG** 1998 Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem J* 334 (Pt 1):261-267
 19. **Patel J, Wang X, Proud CG** 2001 Glucose exerts a permissive effect on the regulation of the initiation factor 4E binding protein 4E-BP1. *Biochem J* 358:497-503
 20. **Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P, Meyuhas O** 2005 Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* 19:2199-2211

21. **Wen HY, Abbasi S, Kellems RE, Xia Y** 2005 mTOR: a placental growth signaling sensor. *Placenta* 26 Suppl A:S63-69
22. **Martin PM, Sutherland AE** 2001 Exogenous amino acids regulate trophoctoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol* 240:182-193
23. **Guillomot M, Flechon JE, Leroy F** 1993 Blastocyst development and implantation. In: C Thibault, MC Levasseur & RHF Hunter (eds.), *Reproduction in Mammals and Man*, Paris: Ellipses, pp. 387-411.
24. **Guillomot M** 1995 Cellular interactions during implantation in domestic ruminants. *Journal of Reproduction and Fertility Supplement*:39-51
25. **Wooding FB** 1992 Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta* 13:101-113
26. **Abrahamson M, Alvarez-Fernandez M, Nathanson CM** 2003 Cystatins. *Biochem Soc Symp*:179-199
27. **Barcroft LC, Hay-Schmidt A, Caveney A, Gilfoyle E, Overstrom EW, Hyttel P, Watson AJ** 1998 Trophoctoderm differentiation in the bovine embryo: characterization of a polarized epithelium. *J Reprod Fertil* 114:327-339
28. **Bindon BM** 1971 Systematic study of preimplantation stages of pregnancy in the sheep. *Aust J Biol Sci* 24:131-147
29. **Chang MC, Rowson LE** 1965 Fertilization and early development of Dorset Horn sheep in the spring and summer. *Anat Rec* 152:303-316
30. **Rowson LE, Moor RM** 1966 Development of the sheep conceptus during the first fourteen days. *J Anat* 100:777-785
31. **Ziomek CA, Johnson MH** 1980 Cell surface interaction induces polarization of mouse 8-cell blastomeres at compaction. *Cell* 21:935-942
32. **Wintenberger-Torres S, Flechon JE** 1974 Ultrastructural evolution of the trophoblast cells of the pre-implantation sheep blastocyst from day 8 to day 18. *J Anat* 118:143-153
33. **Chang MC** 1952 Development of bovine blastocyst with a note on implantation. *Anat Rec* 113:143-161

34. **Averill RL, Adams CE, Rowson LE** 1955 Transfer of mammalian ova between species. *Nature* 176:167-168
35. **Rowson LE, Moor RM** 1966 Embryo transfer in the sheep: the significance of synchronizing oestrus in the donor and recipient animal. *J Reprod Fertil* 11:207-212
36. **Flechon JE, Guillomot M, Charlier M, Flechon B, Martal J** 1986 Experimental studies on the elongation of the ewe blastocyst. *Reprod Nutr Dev* 26:1017-1024
37. **Heyman Y, Camous S, Fevre J, Meziou W, Martal J** 1984 Maintenance of the corpus luteum after uterine transfer of trophoblastic vesicles to cyclic cows and ewes. *J Reprod Fertil* 70:533-540
38. **Guillomot M, Michel C, Gaye P, Charlier N, Trojan J, Martal J** 1990 Cellular localization of an embryonic interferon, ovine trophoblastin and its mRNA in sheep embryos during early pregnancy. *Biol Cell* 68:205-211
39. **Farin CE, Imakawa K, Roberts RM** 1989 In situ localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. *Mol Endocrinol* 3:1099-1107
40. **Bazer FW** 1992 Mediators of maternal recognition of pregnancy in mammals. *Proc Soc Exp Biol Med* 199:373-384
41. **Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M** 2007 Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. *Reprod Fertil Dev* 19:65-78
42. **Thatcher WW, Hansen PJ, Gross TS, Helmer SD, Plante C, Bazer FW** 1989 Antiluteolytic effects of bovine trophoblast protein-1. *J Reprod Fertil Suppl* 37:91-99
43. **Bazer FW, Spencer TE, Ott TL** 1998 Endocrinology of the transition from recurring estrous cycles to establishment of pregnancy in subprimate mammals. In: Bazer FW (ed), *The Endocrinology of Pregnancy* Totowa, NJ: Humana Press 1-34

44. **Spencer TE, Ott TL, Bazer FW** 1996 tau-Interferon: pregnancy recognition signal in ruminants. *Proc Soc Exp Biol Med* 213:215-229
45. **Ashworth CJ, Bazer FW** 1989 Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone. *Biol Reprod* 40:425-433
46. **Roberts RM, Ealy AD, Alexenko AP, Han CS, Ezashi T** 1999 Trophoblast interferons. *Placenta* 20:259-264
47. **Nephew KP, McClure KE, Ott TL, Dubois DH, Bazer FW, Pope WF** 1991 Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. *Biol Reprod* 44:536-539
48. **Fleming JA, Choi Y, Johnson GA, Spencer TE, Bazer FW** 2001 Cloning of the ovine estrogen receptor-alpha promoter and functional regulation by ovine interferon-tau. *Endocrinology* 142:2879-2887
49. **Wathes DC, Gilbert CL, Ayad VJ** 1993 Interactions between oxytocin, the ovaries, and the reproductive tract in the regulation of fertility in the ewe. *Ann N Y Acad Sci* 689:396-410
50. **Spencer TE, Bazer FW** 1995 Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. *Biol Reprod* 53:1527-1543
51. **Hooper SB, Watkins WB, Thorburn GD** 1986 Oxytocin, oxytocin-associated neurophysin, and prostaglandin F2 alpha concentrations in the utero-ovarian vein of pregnant and nonpregnant sheep. *Endocrinology* 119:2590-2597
52. **Spencer TE, Bazer FW** 1996 Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. *Endocrinology* 137:1144-1147
53. **Stevenson KR, Riley PR, Stewart HJ, Flint AP, Wathes DC** 1994 Localization of oxytocin receptor mRNA in the ovine uterus during the oestrous cycle and early pregnancy. *J Mol Endocrinol* 12:93-105
54. **Lamming GE, Wathes DC, Flint AP, Payne JH, Stevenson KR, Vallet JL** 1995 Local action of trophoblast interferons in suppression of the development of

- oxytocin and oestradiol receptors in ovine endometrium. *J Reprod Fertil* 105:165-175
55. **Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW** 1995 Ovine interferon-tau inhibits estrogen receptor up-regulation and estrogen-induced luteolysis in cyclic ewes. *Endocrinology* 136:4932-4944
 56. **Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW** 1995 Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. *Biol Reprod* 53:732-745
 57. **Spencer TE, Burghardt RC, Johnson GA, Bazer FW** 2004 Conceptus signals for establishment and maintenance of pregnancy. *Anim Reprod Sci* 82-83:537-550
 58. **Hansen TR, Austin KJ, Perry DJ, Pru JK, Teixeira MG, Johnson GA** 1999 Mechanism of action of interferon-tau in the uterus during early pregnancy. *J Reprod Fertil Suppl* 54:329-339
 59. **Nagaoka K, Sakai A, Nojima H, Suda Y, Yokomizo Y, Imakawa K, Sakai S, Christenson RK** 2003 A chemokine, interferon (IFN)-gamma-inducible protein 10 kDa, is stimulated by IFN-tau and recruits immune cells in the ovine endometrium. *Biol Reprod* 68:1413-1421
 60. **Nagaoka K, Nojima H, Watanabe F, Chang KT, Christenson RK, Sakai S, Imakawa K** 2003 Regulation of blastocyst migration, apposition, and initial adhesion by a chemokine, interferon gamma-inducible protein 10 kDa (IP-10), during early gestation. *J Biol Chem* 278:29048-29056
 61. **Spencer TE, Bazer FW** 2002 Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front Biosci* 7:d1879-1898
 62. **Spencer TE, Bartol FF, Bazer FW, Johnson GA, Joyce MM** 1999 Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. *Biol Reprod* 60:241-250
 63. **Spencer TE, Bazer FW** 2004 Uterine and placental factors regulating conceptus growth in domestic animals. *J Anim Sci* 82 E-Suppl:E4-13
 64. **Guillomot M, Flechon JE, Wintenberger-Torres S** 1981 Conceptus attachment in the ewe: an ultrastructural study. *Placenta* 2:169-182

65. **Wimsatt WA** 1950 New histological observations on the placenta of the sheep. *Am J Anat* 87:391-457
66. **Spencer TE, Johnson GA, Burghardt RC, Bazer FW** 2004 Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biol Reprod* 71:2-10
67. **Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K** 2000 Embryo implantation. *Dev Biol* 223:217-237
68. **Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, Bazer FW, Spencer TE** 2001 Developmental biology of uterine glands. *Biol Reprod* 65:1311-1323
69. **Martal J, Chene N, Camous S, Huynh L, Lantier F, Hermier P, L'Haridon R, Charpigny G, Charlier M, Chaouat G** 1997 Recent developments and potentialities for reducing embryo mortality in ruminants: the role of IFN-tau and other cytokines in early pregnancy. *Reprod Fertil Dev* 9:355-380
70. **Spencer TE, Stagg AG, Joyce MM, Jenster G, Wood CG, Bazer FW, Wiley AA, Bartol FF** 1999 Discovery and characterization of endometrial epithelial messenger ribonucleic acids using the ovine uterine gland knockout model. *Endocrinology* 140:4070-4080
71. **Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E** 2002 Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *J Clin Endocrinol Metab* 87:2954-2959
72. **Bartol FF, Wiley AA, Floyd JG, Ott TL, Bazer FW, Gray CA, Spencer TE** 1999 Uterine differentiation as a foundation for subsequent fertility. *J Reprod Fertil Suppl* 54:287-302
73. **Richardson CJ, Broenstrup M, Fingar DC, Julich K, Ballif BA, Gygi S, Blenis J** 2004 SKAR is a specific target of S6 kinase 1 in cell growth control. *Curr Biol* 14:1540-1549
74. **Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ** 1992 Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 359:76-79

75. **Zhu LJ, Bagchi MK, Bagchi IC** 1998 Attenuation of calcitonin gene expression in pregnant rat uterus leads to a block in embryonic implantation. *Endocrinology* 139:330-339
76. **Gray CA, Bartol FF, Taylor KM, Wiley AA, Ramsey WS, Ott TL, Bazer FW, Spencer TE** 2000 Ovine uterine gland knock-out model: effects of gland ablation on the estrous cycle. *Biol Reprod* 62:448-456
77. **Gray CA, Taylor KM, Bazer FW, Spencer TE** 2000 Mechanisms regulating norgestomet inhibition of endometrial gland morphogenesis in the neonatal ovine uterus. *Mol Reprod Dev* 57:67-78
78. **Gray CA, Bazer FW, Spencer TE** 2001 Effects of neonatal progestin exposure on female reproductive tract structure and function in the adult ewe. *Biol Reprod* 64:797-804
79. **Gray CA, Adelson DL, Bazer FW, Burghardt RC, Meeusen EN, Spencer TE** 2004 Discovery and characterization of an epithelial-specific galectin in the endometrium that forms crystals in the trophectoderm. *Proc Natl Acad Sci U S A* 101:7982-7987
80. **Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, Adelson DL, Thomas TL, Bazer FW, Spencer TE** 2006 Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. *Biol Reprod* 74:383-394
81. **Bartol FF, Johnson LL, Floyd JG, Wiley AA, Spencer TE, Buxton DF, Coleman DA** 1995 Neonatal exposure to progesterone and estradiol alters uterine morphology and luminal protein content in adult beef heifers. *Theriogenology* 43:835-844
82. **Bartol FF, Roberts RM, Bazer FW, Thatcher WW** 1985 Characterization of proteins produced in vitro by bovine endometrial explants. *Biol Reprod* 33:745-759
83. **Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW** 1985 Characterization of proteins produced in vitro by periattachment bovine conceptuses. *Biol Reprod* 32:681-693

84. **Lee RS, Wheeler TT, Peterson AJ** 1998 Large-format, two-dimensional polyacrylamide gel electrophoresis of ovine periimplantation uterine luminal fluid proteins: identification of aldose reductase, cytoplasmic actin, and transferrin as conceptus-synthesized proteins. *Biol Reprod* 59:743-752
85. **Fazleabas AT, Miller JB, Verhage HG** 1988 Synthesis and release of estrogen- and progesterone-dependent proteins by the baboon (*Papio anubis*) uterine endometrium. *Biol Reprod* 39:729-736
86. **Kayser JP, Kim JG, Cerny RL, Vallet JL** 2006 Global characterization of porcine intrauterine proteins during early pregnancy. *Reproduction* 131:379-388
87. **MacLaughlin DT, Santoro NF, Bauer HH, Lawrence D, Richardson GS** 1986 Two-dimensional gel electrophoresis of endometrial protein in human uterine fluids: qualitative and quantitative analysis. *Biol Reprod* 34:579-585
88. **Berendt FJ, Frohlich T, Schmidt SE, Reichenbach HD, Wolf E, Arnold GJ** 2005 Holistic differential analysis of embryo-induced alterations in the proteome of bovine endometrium in the preattachment period. *Proteomics* 5:2551-2560
89. **Geisert RD, Thatcher WW, Roberts RM, Bazer FW** 1982 Establishment of pregnancy in the pig: III. Endometrial secretory response to estradiol valerate administered on day 11 of the estrous cycle. *Biol Reprod* 27:957-965
90. **Kwon H, Spencer TE, Bazer FW, Wu G** 2003 Developmental changes of amino acids in ovine fetal fluids. *Biol Reprod* 68:1813-1820
91. **Kwon H, Wu G, Bazer FW, Spencer TE** 2003 Developmental changes in polyamine levels and synthesis in the ovine conceptus. *Biol Reprod* 69:1626-1634
92. **Devreker F, Hardy K, Van den Bergh M, Winston J, Biramane J, Englert Y** 2000 Noninvasive assessment of glucose and pyruvate uptake by human embryos after intracytoplasmic sperm injection and during the formation of pronuclei. *Fertil Steril* 73:947-954
93. **Leese HJ** 1995 Metabolic control during preimplantation mammalian development. *Hum Reprod Update* 1:63-72
94. **Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW** 2009 Select nutrients in the ovine uterine lumen. ii. glucose transporters in the uterus and peri-implantation conceptuses. *Biol Reprod* 80:94-104

95. **Gao H, Wu G, Spencer TE, Johnson GA, Li X, Bazer FW** 2009 Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine luminal flushings of cyclic and pregnant ewes. *Biol Reprod* 80:86-93
96. **Martin PM, Sutherland AE, Van Winkle LJ** 2003 Amino acid transport regulates blastocyst implantation. *Biol Reprod* 69:1101-1108
97. **Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW** 2009 Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses. *Biol Reprod* 80:1196-1208
98. **Liao XH, Majithia A, Huang X, Kimmel AR** 2008 Growth control via TOR kinase signaling, an intracellular sensor of amino acid and energy availability, with crosstalk potential to proline metabolism. *Amino Acids* 35:761-770
99. **Inoki K, Ouyang H, Li Y, Guan KL** 2005 Signaling by target of rapamycin proteins in cell growth control. *Microbiol Mol Biol Rev* 69:79-100
100. **Wullschleger S, Loewith R, Hall MN** 2006 TOR signaling in growth and metabolism. *Cell* 124:471-484
101. **Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K** 2002 Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 110:177-189
102. **Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM** 2002 mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110:163-175
103. **Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, Tempst P, Sabatini DM** 2003 GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell* 11:895-904
104. **Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN** 2002 Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* 10:457-468

105. **Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM** 2004 Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 14:1296-1302
106. **Frias MA, Thoreen CC, Jaffe JD, Schroder W, Sculley T, Carr SA, Sabatini DM** 2006 mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol* 16:1865-1870
107. **Schmelzle T, Hall MN** 2000 TOR, a central controller of cell growth. *Cell* 103:253-262
108. **Sarbassov DD, Ali SM, Sabatini DM** 2005 Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 17:596-603
109. **Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, Ru B, Pan D** 2002 Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat Cell Biol* 4:699-704
110. **Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D** 2003 Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat Cell Biol* 5:578-581
111. **Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW** 2009 Select nutrients in the ovine uterine lumen. VI. Expression of FK506-binding protein 12-rapamycin complex-associated protein 1 (FRAP1) and regulators and effectors of mTORC1 and mTORC2 complexes in ovine uteri and conceptuses. *Biol Reprod* 81:87-100
112. **Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J** 2003 Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr Biol* 13:1259-1268
113. **Nielsen FC, Ostergaard L, Nielsen J, Christiansen J** 1995 Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. *Nature* 377:358-362
114. **Kimball SR, Shantz LM, Horetsky RL, Jefferson LS** 1999 Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. *J Biol Chem* 274:11647-11652

115. **Jefferies HB, Reinhard C, Kozma SC, Thomas G** 1994 Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proc Natl Acad Sci U S A* 91:4441-4445
116. **Wathes DC, Reynolds TS, Robinson RS, Stevenson KR** 1998 Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *J Dairy Sci* 81:1778-1789
117. **Zhou J, Bondy C** 1992 Insulin-like growth factor-II and its binding proteins in placental development. *Endocrinology* 131:1230-1240
118. **Ohlsson R, Larsson E, Nilsson O, Wahlstrom T, Sundstrom P** 1989 Blastocyst implantation precedes induction of insulin-like growth factor II gene expression in human trophoblasts. *Development* 106:555-559
119. **Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE** 2004 Maternal nutrition and fetal development. *J Nutr* 134:2169-2172
120. **Zhou QL, Jiang ZY, Holik J, Chawla A, Hagan GN, Leszyk J, Czech MP** 2008 Akt substrate TBC1D1 regulates GLUT1 expression through the mTOR pathway in 3T3-L1 adipocytes. *Biochem J* 411:647-655
121. **Buller CL, Loberg RD, Fan MH, Zhu Q, Park JL, Vesely E, Inoki K, Guan KL, Brosius FC, 3rd** 2008 A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression. *Am J Physiol Cell Physiol* 295:C836-843
122. **Schmid H, Bertoluci M, Coimbra TM** 2008 Glucose transporter 12 and mammalian target of rapamycin complex 1 signaling: a new target for diabetes-induced renal injury? *Endocrinology* 149:913-916
123. **Fuchs BC, Finger RE, Onan MC, Bode BP** 2007 ASCT2 silencing regulates mammalian target-of-rapamycin growth and survival signaling in human hepatoma cells. *Am J Physiol Cell Physiol* 293:C55-63
124. **Fuchs BC, Bode BP** 2005 Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? *Semin Cancer Biol* 15:254-266
125. **van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, van den Ouweland A, Halley D, Young J, Burley M, Jeremiah S, Woodward K, Nahmias J, Fox M, Ekong R, Osborne J, Wolfe J, Povey S,**

- Snell RG, Cheadle JP, Jones AC, Tachataki M, Ravine D, Sampson JR, Reeve MP, Richardson P, Wilmer F, Munro C, Hawkins TL, Sepp T, Ali JB, Ward S, Green AJ, Yates JR, Kwiatkowska J, Henske EP, Short MP, Haines JH, Jozwiak S, Kwiatkowski DJ** 1997 Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* 277:805-808
126. **Gingras AC, Raught B, Sonenberg N** 2001 Regulation of translation initiation by FRAP/mTOR. *Genes Dev* 15:807-826
 127. **Daughaday WH, Rotwein P** 1989 Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr Rev* 10:68-91
 128. **Adamo ML, Neuenschwander S, LeRoith D, Roberts CT, Jr.** 1993 Structure, expression, and regulation of the IGF-I gene. *Adv Exp Med Biol* 343:1-11
 129. **LeRoith D, Roberts CT, Jr.** 1993 Insulin-like growth factors. *Ann N Y Acad Sci* 692:1-9
 130. **Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, et al.** 1986 Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J* 5:2503-2512
 131. **Siddle K, Urso B, Niesler CA, Cope DL, Molina L, Surinya KH, Soos MA** 2001 Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors. *Biochem Soc Trans* 29:513-525
 132. **White MF** 2002 IRS proteins and the common path to diabetes. *Am J Physiol Endocrinol Metab* 283:E413-422
 133. **D'Ercole AJ** 1987 Somatomedins/insulin-like growth factors and fetal growth. *J Dev Physiol* 9:481-495
 134. **Gluckman PD, Morel PC, Ambler GR, Breier BH, Blair HT, McCutcheon SN** 1992 Elevating maternal insulin-like growth factor-I in mice and rats alters the pattern of fetal growth by removing maternal constraint. *J Endocrinol* 134:R1-3
 135. **DeChiara TM, Efstratiadis A, Robertson EJ** 1990 A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* 345:78-80

136. **Baker J, Liu JP, Robertson EJ, Efstratiadis A** 1993 Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75:73-82
137. **Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A** 1993 Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75:59-72
138. **Perks CM, Peters AR, Wathes DC** 1999 Follicular and luteal expression of insulin-like growth factors I and II and the type 1 IGF receptor in the bovine ovary. *J Reprod Fertil* 116:157-165
139. **Watson AJ, Hogan A, Hahnel A, Wiemer KE, Schultz GA** 1992 Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Mol Reprod Dev* 31:87-95
140. **Geisert RD, Lee CY, Simmen FA, Zavy MT, Fliss AE, Bazer FW, Simmen RC** 1991 Expression of messenger RNAs encoding insulin-like growth factor-I, -II, and insulin-like growth factor binding protein-2 in bovine endometrium during the estrous cycle and early pregnancy. *Biol Reprod* 45:975-983
141. **Kirby CJ, Thatcher WW, Collier RJ, Simmen FA, Lucy MC** 1996 Effects of growth hormone and pregnancy on expression of growth hormone receptor, insulin-like growth factor-I, and insulin-like growth factor binding protein-2 and -3 genes in bovine uterus, ovary, and oviduct. *Biol Reprod* 55:996-1002
142. **Robinson RS, Mann GE, Gadd TS, Lamming GE, Wathes DC** 2000 The expression of the IGF system in the bovine uterus throughout the oestrous cycle and early pregnancy. *J Endocrinol* 165:231-243
143. **Ko Y, Lee CY, Ott TL, Davis MA, Simmen RC, Bazer FW, Simmen FA** 1991 Insulin-like growth factors in sheep uterine fluids: concentrations and relationship to ovine trophoblast protein-1 production during early pregnancy. *Biol Reprod* 45:135-142
144. **Stevenson KR, Gilmour RS, Wathes DC** 1994 Localization of insulin-like growth factor-I (IGF-I) and -II messenger ribonucleic acid and type 1 IGF receptors in the ovine uterus during the estrous cycle and early pregnancy. *Endocrinology* 134:1655-1664

145. **Perks CM, Denning-Kendall PA, Gilmour RS, Wathes DC** 1995 Localization of messenger ribonucleic acids for insulin-like growth factor I (IGF-I), IGF-II, and the type 1 IGF receptor in the ovine ovary throughout the estrous cycle. *Endocrinology* 136:5266-5273
146. **Stevenson KR, Wathes DC** 1996 Insulin-like growth factors and their binding proteins in the ovine oviduct during the oestrous cycle. *J Reprod Fertil* 108:31-40
147. **Reynolds TS, Stevenson KR, Wathes DC** 1997 Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology* 138:886-897
148. **Sterle JA, Boyd C, Peacock JT, Koenigsfeld AT, Lamberson WR, Gerrard DE, Lucy MC** 1998 Insulin-like growth factor (IGF)-I, IGF-II, IGF-binding protein-2 and pregnancy-associated glycoprotein mRNA in pigs with somatotropin-enhanced fetal growth. *J Endocrinol* 159:441-450
149. **Lee RS, Depree KM, Davey HW** 2002 The sheep (*Ovis aries*) H19 gene: genomic structure and expression patterns, from the preimplantation embryo to adulthood. *Gene* 301:67-77
150. **Qiu Q, Basak A, Mbikay M, Tsang BK, Gruslin A** 2005 Role of pro-IGF-II processing by proprotein convertase 4 in human placental development. *Proc Natl Acad Sci U S A* 102:11047-11052
151. **O'Dell SD, Day IN** 1998 Insulin-like growth factor II (IGF-II). *Int J Biochem Cell Biol* 30:767-771
152. **DeChiara TM, Robertson EJ, Efstratiadis A** 1991 Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 64:849-859
153. **Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, Reik W** 2002 Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* 417:945-948
154. **Fowden AL** 2003 The insulin-like growth factors and feto-placental growth. *Placenta* 24:803-812
155. **Sibley CP, Coan PM, Ferguson-Smith AC, Dean W, Hughes J, Smith P, Reik W, Burton GJ, Fowden AL, Constancia M** 2004 Placental-specific insulin-like

- growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. *Proc Natl Acad Sci U S A* 101:8204-8208
156. **Han VK, Carter AM** 2000 Spatial and temporal patterns of expression of messenger RNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals. *Placenta* 21:289-305
 157. **Smith GC, Stenhouse EJ, Crossley JA, Aitken DA, Cameron AD, Connor JM** 2002 Early pregnancy levels of pregnancy-associated plasma protein a and the risk of intrauterine growth restriction, premature birth, preeclampsia, and stillbirth. *J Clin Endocrinol Metab* 87:1762-1767
 158. **Taylor KM, Chen C, Gray CA, Bazer FW, Spencer TE** 2001 Expression of messenger ribonucleic acids for fibroblast growth factors 7 and 10, hepatocyte growth factor, and insulin-like growth factors and their receptors in the neonatal ovine uterus. *Biol Reprod* 64:1236-1246
 159. **Hayashi K, Carpenter KD, Welsh TH, Jr., Burghardt RC, Spicer LJ, Spencer TE** 2005 The IGF system in the neonatal ovine uterus. *Reproduction* 129:337-347
 160. **Watson AJ, Westhusin ME, Winger QA** 1999 IGF paracrine and autocrine interactions between conceptus and oviduct. *J Reprod Fertil Suppl* 54:303-315
 161. **Fowden AL, Sibley C, Reik W, Constancia M** 2006 Imprinted genes, placental development and fetal growth. *Horm Res* 65 Suppl 3:50-58
 162. **Franzen A, Heinegard D** 1985 Isolation and characterization of two sialoproteins present only in bone calcified matrix. *Biochem J* 232:715-724
 163. **Oldberg A, Franzen A, Heinegard D** 1986 Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc Natl Acad Sci U S A* 83:8819-8823
 164. **Sodek J, Ganss B, McKee MD** 2000 Osteopontin. *Crit Rev Oral Biol Med* 11:279-303
 165. **Butler WT** 1995 Structural and functional domains of osteopontin. *Ann N Y Acad Sci* 760:6-11
 166. **Butler WT, A.L. Ridall, and M.D. McKee** 1996 Osteopontin. *Principles of Bone Biology*: New York: Academic Press.

167. **Johnson GA, Burghardt RC, Bazer FW, Spencer TE** 2003 Osteopontin: roles in implantation and placentation. *Biol Reprod* 69:1458-1471
168. **Bayless KJ, Meininger GA, Scholtz JM, Davis GE** 1998 Osteopontin is a ligand for the $\alpha 4 \beta 1$ integrin. *J Cell Sci* 111 (Pt 9):1165-1174
169. **Smith LL, Cheung HK, Ling LE, Chen J, Sheppard D, Pytela R, Giachelli CM** 1996 Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by $\alpha 9 \beta 1$ integrin. *J Biol Chem* 271:28485-28491
170. **Weber GF, Ashkar S, Cantor H** 1997 Interaction between CD44 and osteopontin as a potential basis for metastasis formation. *Proc Assoc Am Physicians* 109:1-9
171. **Weber GF, Ashkar S, Glimcher MJ, Cantor H** 1996 Receptor-ligand interaction between CD44 and osteopontin (Eta-1). *Science* 271:509-512
172. **Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW** 1999 Ovine osteopontin: II. Osteopontin and $\alpha (v) \beta (3)$ integrin expression in the uterus and conceptus during the periimplantation period. *Biol Reprod* 61:892-899
173. **Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M, Yuan L, Fritz MA, Lessey B** 2002 Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Mol Hum Reprod* 8:871-879
174. **Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S, Oehninger S** 2005 In search of candidate genes critically expressed in the human endometrium during the window of implantation. *Hum Reprod* 20:2104-2117
175. **Apparao KB, Illera MJ, Beyler SA, Olson GE, Osteen KG, Corjay MH, Boggess K, Lessey BA** 2003 Regulated expression of osteopontin in the peri-implantation rabbit uterus. *Biol Reprod* 68:1484-1490
176. **Joyce MM, Gonzalez JF, Lewis S, Woldesenbet S, Burghardt RC, Newton GR, Johnson GA** 2005 Caprine uterine and placental osteopontin expression is distinct among epitheliochorial implanting species. *Placenta* 26:160-170

177. **Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA, Giudice LC** 2002 Global gene profiling in human endometrium during the window of implantation. *Endocrinology* 143:2119-2138
178. **Yokasaki Y, Sheppard D** 2000 Mapping of the cryptic integrin-binding site in osteopontin suggests a new mechanism by which thrombin can regulate inflammation and tissue repair. *Trends Cardiovasc Med* 10:155-159
179. **Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW, Sun J** 1994 Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil Steril* 62:497-506
180. **Bowen JA, Bazer FW, Burghardt RC** 1997 Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophoctoderm in vitro. *Biol Reprod* 56:409-415
181. **Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE, Burghardt RC** 2001 Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. *Biol Reprod* 65:820-828
182. **Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW** 2002 Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells Tissues Organs* 172:202-217
183. **Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA, Bazer FW** 2002 Analysis of osteopontin at the maternal-placental interface in pigs. *Biol Reprod* 66:718-725
184. **Goldsmith HL, Labrosse JM, McIntosh FA, Maenpaa PH, Kaartinen MT, McKee MD** 2002 Homotypic interactions of soluble and immobilized osteopontin. *Ann Biomed Eng* 30:840-850
185. **Burghardt RC, Burghardt JR, Taylor JD, 2nd, Reeder AT, Nguen BT, Spencer TE, Bayless KJ, Johnson GA** 2009 Enhanced focal adhesion assembly reflects increased mechanosensation and mechanotransduction at maternal-conceptus interface and uterine wall during ovine pregnancy. *Reproduction* 137:567-582
186. **Weintraub AS, Lin X, Itskovich VV, Aguinaldo JG, Chaplin WF, Denhardt DT, Fayad ZA** 2004 Prenatal detection of embryo resorption in osteopontin-

- deficient mice using serial noninvasive magnetic resonance microscopy. *Pediatr Res* 55:419-424
187. **Johnson GA, Spencer TE, Burghardt RC, Bazer FW** 1999 Ovine osteopontin: I. Cloning and expression of messenger ribonucleic acid in the uterus during the periimplantation period. *Biol Reprod* 61:884-891
 188. **Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA, Bazer FW** 2000 Progesterone modulation of osteopontin gene expression in the ovine uterus. *Biol Reprod* 62:1315-1321
 189. **Johnson GA, Stewart MD, Gray CA, Choi Y, Burghardt RC, Yu-Lee LY, Bazer FW, Spencer TE** 2001 Effects of the estrous cycle, pregnancy, and interferon tau on 2',5'-oligoadenylate synthetase expression in the ovine uterus. *Biol Reprod* 64:1392-1399
 190. **Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M** 1996 Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the α v β 3 integrin, osteopontin, and thrombin. *Am J Pathol* 149:293-305
 191. **Lessey BA, Damjanovich L, Coutifaris C, Castelbaum A, Albelda SM, Buck CA** 1992 Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. *J Clin Invest* 90:188-195
 192. **Dunlap KA, Erikson DW, Burghardt RC, White FJ, Reed KM, Farmer JL, Spencer TE, Magness RR, Bazer FW, Bayless KJ, Johnson GA** 2008 Progesterone and placentation increase secreted phosphoprotein one (SPP1 or osteopontin) in uterine glands and stroma for histotrophic and hematotrophic support of ovine pregnancy. *Biol Reprod* 79:983-990
 193. **Apparao KB, Murray MJ, Fritz MA, Meyer WR, Chambers AF, Truong PR, Lessey BA** 2001 Osteopontin and its receptor α v β (3) integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. *J Clin Endocrinol Metab* 86:4991-5000
 194. **Jobgen W, Meininger CJ, Jobgen SC, Li P, Lee MJ, Smith SB, Spencer TE, Fried SK, Wu G** 2009 Dietary L-arginine supplementation reduces white fat gain

- and enhances skeletal muscle and brown fat masses in diet-induced obese rats. *J Nutr* 139:230-237
195. **Sooranna SR, Morris NH, Steer PJ** 1995 Placental nitric oxide metabolism. *Reprod Fertil Dev* 7:1525-1531
 196. **Zhao YC, Chi YJ, Yu YS, Liu JL, Su RW, Ma XH, Shan CH, Yang ZM** 2008 Polyamines are essential in embryo implantation: expression and function of polyamine-related genes in mouse uterus during peri-implantation period. *Endocrinology* 149:2325-2332
 197. **Wu G, Bazer FW, Wallace JM, Spencer TE** 2006 Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci* 84:2316-2337
 198. **Meier P, Teng C, Battaglia FC, Meschia G** 1981 The rate of amino acid nitrogen and total nitrogen accumulation in the fetal lamb. *Proc Soc Exp Biol Med* 167:463-468
 199. **Wu G, Ott TL, Knabe DA, Bazer FW** 1999 Amino acid composition of the fetal pig. *J Nutr* 129:1031-1038
 200. **Sparks JW, Pegorier JP, Girard J, Battaglia FC** 1981 Substrate concentration changes during pregnancy in the guinea pig studied under unstressed steady state conditions. *Pediatr Res* 15:1340-1344
 201. **Zeng X, Wang F, Fan X, Yang W, Zhou B, Li P, Yin Y, Wu G, Wang J** 2008 Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. *J Nutr* 138:1421-1425
 202. **Mateo RD, Wu G, Bazer FW, Park JC, Shinzato I, Kim SW** 2007 Dietary L-arginine supplementation enhances the reproductive performance of gilts. *J Nutr* 137:652-656
 203. **Wu G BF, Datta S et al** 2008 Intrauterine growth retardation in livestock: implications, mechanisms and solutions. *Arch Fur Tierzucht-Arch Anim Breed* 51:4-10
 204. **Igarashi K, Kashiwagi K** 2000 Polyamines: mysterious modulators of cellular functions. *Biochem Biophys Res Commun* 271:559-564

205. **Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, Smith SB, Spencer TE, Yin Y** 2009 Arginine metabolism and nutrition in growth, health and disease. *Amino Acids* 37:153-168
206. **Wu G, Bazer FW, Datta S, Johnson GA, Li P, Satterfield MC, Spencer TE** 2008 Proline metabolism in the conceptus: implications for fetal growth and development. *Amino Acids* 35:691-702
207. **Greenberg SS, Lancaster JR, Xie J, Sarphie TG, Zhao X, Hua L, Freeman T, Kapusta DR, Giles TD, Powers DR** 1997 Effects of NO synthase inhibitors, arginine-deficient diet, and amiloride in pregnant rats. *Am J Physiol* 273:R1031-1045
208. **Lassala A, Bazer FW, Cudd TA, Li P, Li X, Satterfield MC, Spencer TE, Wu G** 2009 Intravenous administration of L-citrulline to pregnant ewes is more effective than L-arginine for increasing arginine availability in the fetus. *J Nutr* 139:660-665
209. **Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G** 2006 Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. *J Nutr Biochem* 17:571-588
210. **Van Winkle LJ, Tesch JK, Shah A, Campione AL** 2006 System B₀,+ amino acid transport regulates the penetration stage of blastocyst implantation with possible long-term developmental consequences through adulthood. *Hum Reprod Update* 12:145-157
211. **Finch AM, Yang LG, Nwagwu MO, Page KR, McArdle HJ, Ashworth CJ** 2004 Placental transport of leucine in a porcine model of low birth weight. *Reproduction* 128:229-235
212. **Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW** 2009 Select Nutrients in the Ovine Uterine Lumen. IV. Expression of Neutral and Acidic Amino Acid Transporters in Ovine Uteri and Periimplantation Conceptuses. *Biol Reprod* 80(6):1196-1208
213. **Wu G, Knabe DA, Yan W, Flynn NE** 1995 Glutamine and glucose metabolism in enterocytes of the neonatal pig. *Am J Physiol* 268:R334-342

214. **Krebs HA, Baverel G, Lund P** 1980 Effect of bicarbonate on glutamine metabolism. *Int J Biochem* 12:69-73
215. **Bell AW, Kennaugh JM, Battaglia FC, Meschia G** 1989 Uptake of amino acids and ammonia at mid-gestation by the fetal lamb. *Q J Exp Physiol* 74:635-643
216. **Vaughn PR, Lobo C, Battaglia FC, Fennessey PV, Wilkening RB, Meschia G** 1995 Glutamine-glutamate exchange between placenta and fetal liver. *Am J Physiol* 268:E705-711
217. **Petters RM, Johnson BH, Reed ML, Archibong AE** 1990 Glucose, glutamine and inorganic phosphate in early development of the pig embryo *in vitro*. *J Reprod Fertil* 89:269-275
218. **Atlante A, Calissano P, Bobba A, Giannattasio S, Marra E, Passarella S** 2001 Glutamate neurotoxicity, oxidative stress and mitochondria. *FEBS Lett* 497:1-5
219. **Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, Yang H, Hild M, Kung C, Wilson C, Myer VE, MacKeigan JP, Porter JA, Wang YK, Cantley LC, Finan PM, Murphy LO** 2009 Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* 136:521-534
220. **Moley KH, Chi MM, Knudson CM, Korsmeyer SJ, Mueckler MM** 1998 Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nat Med* 4:1421-1424
221. **Leese HJ, Barton AM** 1984 Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil* 72:9-13
222. **Pantaleon M, Harvey MB, Pascoe WS, James DE, Kaye PL** 1997 Glucose transporter GLUT3: ontogeny, targeting, and role in the mouse blastocyst. *Proc Natl Acad Sci U S A* 94:3795-3800
223. **Bazer FW, Thatcher WW, Matinat-Botte F, Terqui M, Lacroix MC, Bernard S, Revault M, Dubois DH** 1991 Composition of uterine flushings from Large White and prolific Chinese Meishan gilts. *Reprod Fertil Dev* 3:51-60
224. **Schneider NO, Calderon RO, de Fabro SP** 1981 Isolation and characterization of cell membranes from human placenta. *Acta Physiol Lat Am* 31:283-289
225. **Wood IS, Trayhurn P** 2003 Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *Br J Nutr* 89:3-9

- 226. **Riley JK, Moley KH** 2006 Glucose utilization and the PI3-K pathway: mechanisms for cell survival in preimplantation embryos. *Reproduction* 131:823-835
- 227. **Scheepers A, Joost HG, Schurmann A** 2004 The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. *JPEN J Parenter Enteral Nutr* 28:364-371
- 228. **Gardner DK, Lane M, Calderon I, Leeton J** 1996 Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 65:349-353
- 229. **Sara VR, Hall K** 1990 Insulin-like growth factors and their binding proteins. *Physiol Rev* 70:591-614
- 230. **Rechler MM, Nissley SP** 1985 The nature and regulation of the receptors for insulin-like growth factors. *Annu Rev Physiol* 47:425-442
- 231. **Jones JI, Clemmons DR** 1995 Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev* 16:3-34
- 232. **Daoud G, Amyot M, Rassart E, Masse A, Simoneau L, Lafond J** 2005 ERK1/2 and p38 regulate trophoblasts differentiation in human term placenta. *J Physiol* 566:409-423
- 233. **Dunlap KA, Palmarini M, Adelson DL, Spencer TE** 2005 Sheep endogenous betaretroviruses (enJSRVs) and the hyaluronidase 2 (HYAL2) receptor in the ovine uterus and conceptus. *Biol Reprod* 73:271-279
- 234. **Stewart DM, Johnson GA, Vyhlidal CA, Burghardt RC, Safe SH, Yu-Lee LY, Bazer FW, Spencer TE** 2001 Interferon-tau activates multiple signal transducer and activator of transcription proteins and has complex effects on interferon-responsive gene transcription in ovine endometrial epithelial cells. *Endocrinology* 142:98-107
- 235. **Stewart MD, Johnson GA, Bazer FW, Spencer TE** 2001 Interferon-tau (IFNtau) regulation of IFN-stimulated gene expression in cell lines lacking specific IFN-signaling components. *Endocrinology* 142:1786-1794

236. **Hayashi K, Burghardt RC, Bazer FW, Spencer TE** 2007 WNTs in the ovine uterus: potential regulation of periimplantation ovine conceptus development. *Endocrinology* 148:3496-3506
237. **Culjkovic B, Topisirovic I, Borden KL** 2007 Controlling gene expression through RNA regulons: the role of the eukaryotic translation initiation factor eIF4E. *Cell Cycle* 6:65-69
238. **Hoffmann C, Bazer FW, Klug J, Aupperle H, Ellenberger C, Schoon HA** 2008 Immunohistochemical and histochemical identification of proteins and carbohydrates in the equine endometrium Expression patterns for mares suffering from endometrosis. *Theriogenology* 71(2):264-274
239. **Boshier DP** 1969 A histological and histochemical examination of implantation and early placentome formation in sheep. *J Reprod Fertil* 19:51-61
240. **Giudice LC, Irwin JC** 1999 Roles of the insulinlike growth factor family in nonpregnant human endometrium and at the decidual: trophoblast interface. *Semin Reprod Endocrinol* 17:13-21
241. **Zhou J, Dsupin BA, Giudice LC, Bondy CA** 1994 Insulin-like growth factor system gene expression in human endometrium during the menstrual cycle. *J Clin Endocrinol Metab* 79:1723-1734
242. **Nayak NR, Giudice LC** 2003 Comparative biology of the IGF system in endometrium, decidua, and placenta, and clinical implications for foetal growth and implantation disorders. *Placenta* 24:281-296
243. **Norstedt G, Levinovitz A, Eriksson H** 1989 Regulation of uterine insulin-like growth factor I mRNA and insulin-like growth factor II mRNA by estrogen in the rat. *Acta Endocrinol (Copenh)* 120:466-472
244. **Simmen FA, Simmen RC, Geisert RD, Martinat-Botte F, Bazer FW, Terqui M** 1992 Differential expression, during the estrous cycle and pre- and postimplantation conceptus development, of messenger ribonucleic acids encoding components of the pig uterine insulin-like growth factor system. *Endocrinology* 130:1547-1556
245. **Lee JE, Pinter J, Efstratiadis A** 1990 Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. *Development* 110:151-159

246. **Adashi EY, Resnick CE, Rosenfeld RG** 1990 Insulin-like growth factor-I (IGF-I) and IGF-II hormonal action in cultured rat granulosa cells: mediation via type I but not type II IGF receptors. *Endocrinology* 126:216-222
247. **Lacroix MC, Servely JL, Kann G** 1995 IGF-I and IGF-II receptors in the sheep placenta: evolution during the course of pregnancy. *J Endocrinol* 144:179-191
248. **Rechler MM, Brown AL** 1992 Insulin-like growth factor binding proteins: gene structure and expression. *Growth Regul* 2:55-68
249. **Ferry RJ, Jr., Katz LE, Grimberg A, Cohen P, Weinzimer SA** 1999 Cellular actions of insulin-like growth factor binding proteins. *Horm Metab Res* 31:192-202
250. **Molnar P, Murphy LJ** 1994 Effects of oestrogen on rat uterine expression of insulin-like growth factor-binding proteins. *J Mol Endocrinol* 13:59-67
251. **Osgerby JC, Gadd TS, Wathes DC** 1999 Expression of insulin-like growth factor binding protein-1 (IGFBP-1) mRNA in the ovine uterus throughout the oestrous cycle and early pregnancy. *J Endocrinol* 162:279-287
252. **Gadd TS, Osgerby JC, Wathes DC** 2002 Regulation of insulin-like growth factor binding protein-6 expression in the reproductive tract throughout the estrous cycle and during the development of the placenta in the ewe. *Biol Reprod* 67:1756-1762
253. **Gadd TS, Osgerby JC, Wathes DC** 2000 Regulation and localization of insulin-like growth factor binding protein-5 gene expression in the uterus and placenta of the cyclic and early pregnant ewe. *Biol Reprod* 62:1415-1421
254. **Toyofuku A, Hara T, Taguchi T, Katsura Y, Ohama K, Kudo Y** 2006 Cyclic and characteristic expression of phosphorylated Akt in human endometrium and decidual cells *in vivo* and *in vitro*. *Hum Reprod* 21:1122-1128
255. **Gingras AC, Raught B, Sonenberg N** 2004 mTOR signaling to translation. *Curr Top Microbiol Immunol* 279:169-197
256. **Widmann C, Gibson S, Jarpe MB, Johnson GL** 1999 Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 79:143-180

257. **Wang Y, Wang F, Sun T, Trostinskaia A, Wygle D, Puscheck E, Rappolee DA** 2004 Entire mitogen activated protein kinase (MAPK) pathway is present in preimplantation mouse embryos. *Dev Dyn* 231:72-87
258. **Fernandez-Serra M, Consales C, Livigni A, Arnone MI** 2004 Role of the ERK-mediated signaling pathway in mesenchyme formation and differentiation in the sea urchin embryo. *Dev Biol* 268:384-402
259. **Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, Shen MM** 2000 Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. *Proc Natl Acad Sci U S A* 97:10454-10459
260. **Hay N, Sonenberg N** 2004 Upstream and downstream of mTOR. *Genes Dev* 18:1926-1945
261. **Aplin JD, Kimber SJ** 2004 Trophoblast-uterine interactions at implantation. *Reprod Biol Endocrinol* 2:48-60
262. **Lessey BA, Castelbaum AJ** 2002 Integrins and implantation in the human. *Rev Endocr Metab Disord* 3:107-117
263. **Geisert RD, Yelich JV** 1997 Regulation of conceptus development and attachment in pigs. *J Reprod Fertil Suppl* 52:133-149
264. **Erikson DW, Burghardt RC, Bayless KJ, Johnson GA** 2009 Secreted Phosphoprotein 1 (SPP1, Osteopontin) Binds to Integrin Alpha5beta1 on Porcine Trophectoderm Cells and Integrin Alpha5beta3 on Uterine Luminal Epithelial Cells, and Promotes Trophectoderm Cell Adhesion and Migration. *Biol Reprod* 81(5):814-825
265. **Al-Shami R, Sorensen ES, Ek-Rylander B, Andersson G, Carson DD, Farach-Carson MC** 2005 Phosphorylated osteopontin promotes migration of human choriocarcinoma cells via a p70 S6 kinase-dependent pathway. *J Cell Biochem* 94:1218-1233
266. **Liu L, Chen L, Chung J, Huang S** 2008 Rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins. *Oncogene* 27:4998-5010
267. **Spencer TE, Stagg AG, Ott TL, Johnson GA, Ramsey WS, Bazer FW** 1999 Differential effects of intrauterine and subcutaneous administration of

- recombinant ovine interferon tau on the endometrium of cyclic ewes. *Biol Reprod* 61:464-470
268. **Kim J, Song G, Gao H, Farmer JL, Satterfield MC, Burghardt RC, Wu G, Johnson GA, Spencer TE, Bazer FW** 2008 Insulin-like growth factor II activates phosphatidylinositol 3-kinase-protooncogenic protein kinase 1 and mitogen-activated protein kinase cell Signaling pathways, and stimulates migration of ovine trophectoderm cells. *Endocrinology* 149:3085-3094
 269. **Bayless KJ, Davis GE, Meininger GA** 1997 Isolation and biological properties of osteopontin from bovine milk. *Protein Expr Purif* 9:309-314
 270. **Jaeger LA, Spiegel AK, Ing NH, Johnson GA, Bazer FW, Burghardt RC** 2005 Functional effects of transforming growth factor beta on adhesive properties of porcine trophectoderm. *Endocrinology* 146:3933-3942
 271. **Mosmann T** 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55-63
 272. **Davis GE, Camarillo CW** 1993 Regulation of integrin-mediated myeloid cell adhesion to fibronectin: influence of disulfide reducing agents, divalent cations and phorbol ester. *J Immunol* 151:7138-7150
 273. **Bayless KJ, Davis GE** 2003 Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices. *Biochem Biophys Res Commun* 312:903-913
 274. **Humphries JD, Byron A, Humphries MJ** 2006 Integrin ligands at a glance. *J Cell Sci* 119:3901-3903
 275. **Yokosaki Y, Tanaka K, Higashikawa F, Yamashita K, Eboshida A** 2005 Distinct structural requirements for binding of the integrins α v β 6, α v β 3, α v β 5, α 5 β 1 and α 9 β 1 to osteopontin. *Matrix Biol* 24:418-427
 276. **Okina E, Manon-Jensen T, Whiteford JR, Couchman JR** 2009 Syndecan proteoglycan contributions to cytoskeletal organization and contractility. *Scand J Med Sci Sports* 19:479-489

277. **Kon S, Ikesue M, Kimura C, Aoki M, Nakayama Y, Saito Y, Kurotaki D, Diao H, Matsui Y, Segawa T, Maeda M, Kojima T, Uede T** 2008 Syndecan-4 protects against osteopontin-mediated acute hepatic injury by masking functional domains of osteopontin. *J Exp Med* 205:25-33
278. **Shahbazian D, Roux PP, Mieulet V, Cohen MS, Raught B, Taunton J, Hershey JW, Blenis J, Pende M, Sonenberg N** 2006 The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. *EMBO J* 25:2781-2791
279. **Gailit J, Ruoslahti E** 1988 Regulation of the fibronectin receptor affinity by divalent cations. *J Biol Chem* 263:12927-12932
280. **Kirchhofer D, Grzesiak J, Pierschbacher MD** 1991 Calcium as a potential physiological regulator of integrin-mediated cell adhesion. *J Biol Chem* 266:4471-4477
281. **Tadokoro S, Shattil SJ, Eto K, Tai V, Liddington RC, de Pereda JM, Ginsberg MH, Calderwood DA** 2003 Talin binding to integrin beta tails: a final common step in integrin activation. *Science* 302:103-106
282. **Ireland JJ, Roberts RM, Palmer GH, Bauman DE, Bazer FW** 2008 A commentary on domestic animals as dual-purpose models that benefit agricultural and biomedical research. *J Anim Sci* 86:2797-2805
283. **Chen CS, Alonso JL, Ostuni E, Whitesides GM, Ingber DE** 2003 Cell shape provides global control of focal adhesion assembly. *Biochem Biophys Res Commun* 307:355-361
284. **Wang J, Ito T, Udaka N, Okudela K, Yazawa T, Kitamura H** 2005 PI3K-AKT pathway mediates growth and survival signals during development of fetal mouse lung. *Tissue Cell* 37:25-35
285. **Tokuda H, Hatakeyama D, Shibata T, Akamatsu S, Oiso Y, Kozawa O** 2003 p38 MAP kinase regulates BMP-4-stimulated VEGF synthesis via p70 S6 kinase in osteoblasts. *Am J Physiol Endocrinol Metab* 284:E1202-1209
286. **Ingber DE** 2008 Tensegrity-based mechanosensing from macro to micro. *Prog Biophys Mol Biol* 97:163-179

287. **Wu G** 2009 Amino acids: metabolism, functions, and nutrition. *Amino Acids* 37:1-17
288. **Morris SM, Jr.** 2007 Arginine metabolism: boundaries of our knowledge. *J Nutr* 137:1602S-1609S
289. **Wang WW, Qiao SY, Li DF** 2009 Amino acids and gut function. *Amino Acids* 37:105-110
290. **Flynn NE, Bird JG, Guthrie AS** 2009 Glucocorticoid regulation of amino acid and polyamine metabolism in the small intestine. *Amino Acids* 37:123-129
291. **Kuhara T, Ikeda S, Ohneda A, Sasaki Y** 1991 Effects of intravenous infusion of 17 amino acids on the secretion of GH, glucagon, and insulin in sheep. *Am J Physiol* 260:E21-26
292. **Van Winkle LJ** 2001 Amino acid transport regulation and early embryo development. *Biol Reprod* 64:1-12
293. **Reynolds LP, Caton JS, Redmer DA, Grazul-Bilska AT, Vonnahme KA, Borowicz PP, Luther JS, Wallace JM, Wu G, Spencer TE** 2006 Evidence for altered placental blood flow and vascularity in compromised pregnancies. *J Physiol* 572:51-58
294. **Joyce JM, Phernetton TM, Magness RR** 2002 Effect of uterine blood flow occlusion on shear stress-mediated nitric oxide production and endothelial nitric oxide synthase expression during ovine pregnancy. *Biol Reprod* 67:320-326
295. **Sladek SM, Magness RR, Conrad KP** 1997 Nitric oxide and pregnancy. *Am J Physiol* 272:R441-463
296. **Fozard JR, Part ML, Prakash NJ, Grove J, Schechter PJ, Sjoerdsma A, Koch-Weser J** 1980 L-Ornithine decarboxylase:an essential role in early mammalian embryogenesis. *Science* 208:505-508
297. **Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW** 2009 Select Nutrients in the Ovine Uterine Lumen. III. Cationic Amino Acid Transporters in the Ovine Uterus and Peri-Implantation Conceptuses. *Biol Reprod* 80:602-609
298. **Satterfield MC, Gao H, Li X, Wu G, Johnson GA, Spencer TE, Bazer FW** Select nutrients and their associated transporters are increased in the ovine uterus following early progesterone administration. *Biol Reprod* 82:224-231

299. **Rasputnig G, Fauler G, Jantscher A, Windischhofer W, Schachl K, Leis HJ** 1999 Colorimetric determination of cell numbers by Janus green staining. *Anal Biochem* 275:74-83
300. **Farmer JL, Burghardt RC, Jousan FD, Hansen PJ, Bazer FW, Spencer TE** 2008 Galectin 15 (LGALS15) functions in trophectoderm migration and attachment. *FASEB J* 22:548-560
301. **Pine R, Canova A, Schindler C** 1994 Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. *EMBO J* 13:158-167
302. **Jefferies HB, Fumagalli S, Dennis PB, Reinhard C, Pearson RB, Thomas G** 1997 Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *EMBO J* 16:3693-3704
303. **Kimball SR, Jefferson LS** 2006 Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. *J Nutr* 136:227S-231S
304. **Ban H, Shigemitsu K, Yamatsuji T, Haisa M, Nakajo T, Takaoka M, Nobuhisa T, Gunduz M, Tanaka N, Naomoto Y** 2004 Arginine and Leucine regulate p70 S6 kinase and 4E-BP1 in intestinal epithelial cells. *Int J Mol Med* 13:537-543
305. **Rhoads JM, Liu Y, Niu X, Surendran S, Wu G** 2008 Arginine stimulates cdx2-transformed intestinal epithelial cell migration via a mechanism requiring both nitric oxide and phosphorylation of p70 S6 kinase. *J Nutr* 138:1652-1657
306. **Corl BA, Odle J, Niu X, Moeser AJ, Gatlin LA, Phillips OT, Blikslager AT, Rhoads JM** 2008 Arginine activates intestinal p70(S6k) and protein synthesis in piglet rotavirus enteritis. *J Nutr* 138:24-29
307. **Suryawan A, Jeyapalan AS, Orellana RA, Wilson FA, Nguyen HV, Davis TA** 2008 Leucine stimulates protein synthesis in skeletal muscle of neonatal pigs by enhancing mTORC1 activation. *Am J Physiol Endocrinol Metab* 295:E868-875
308. **Tomiya T, Nishikawa T, Inoue Y, Ohtomo N, Ikeda H, Tejima K, Watanabe N, Tanoue Y, Omata M, Fujiwara K** 2007 Leucine stimulates HGF production

- by hepatic stellate cells through mTOR pathway. *Biochem Biophys Res Commun* 358:176-180
309. **Proud CG** 2009 mTORC1 signalling and mRNA translation. *Biochem Soc Trans* 37:227-231
 310. **Wang X, Proud CG** 2009 Nutrient control of TORC1, a cell-cycle regulator. *Trends Cell Biol* 19:260-267
 311. **Ma XM, Yoon SO, Richardson CJ, Julich K, Blenis J** 2008 SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs. *Cell* 133:303-313
 312. **Rosner M, Hengstschlager M** 2008 Cytoplasmic and nuclear distribution of the protein complexes mTORC1 and mTORC2: rapamycin triggers dephosphorylation and delocalization of the mTORC2 components rictor and sin1. *Hum Mol Genet* 17:2934-2948
 313. **Nakajo T, Yamatsuji T, Ban H, Shigemitsu K, Haisa M, Motoki T, Noma K, Nobuhisa T, Matsuoka J, Gunduz M, Yonezawa K, Tanaka N, Naomoto Y** 2005 Glutamine is a key regulator for amino acid-controlled cell growth through the mTOR signaling pathway in rat intestinal epithelial cells. *Biochem Biophys Res Commun* 326:174-180
 314. **Larson SD, Li J, Chung DH, Evers BM** 2007 Molecular mechanisms contributing to glutamine-mediated intestinal cell survival. *Am J Physiol Gastrointest Liver Physiol* 293:G1262-1271
 315. **Nishikawa T, Tomiya T, Ohtomo N, Inoue Y, Ikeda H, Tejima K, Watanabe N, Tanoue Y, Omata M, Fujiwara K** 2007 Stimulation by glutamine and proline of HGF production in hepatic stellate cells. *Biochem Biophys Res Commun* 363:978-982
 316. **Marc Rhoads J, Wu G** 2009 Glutamine, arginine, and leucine signaling in the intestine. *Amino Acids* 37:111-122
 317. **Suryawan A, Orellana RA, Nguyen HV, Jeyapalan AS, Fleming JR, Davis TA** 2007 Activation by insulin and amino acids of signaling components leading to translation initiation in skeletal muscle of neonatal pigs is developmentally regulated. *Am J Physiol Endocrinol Metab* 293:E1597-1605

318. **Beugnet A, Tee AR, Taylor PM, Proud CG** 2003 Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. *Biochem J* 372:555-566
319. **Christie GR, Hajduch E, Hundal HS, Proud CG, Taylor PM** 2002 Intracellular sensing of amino acids in *Xenopus laevis* oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. *J Biol Chem* 277:9952-9957
320. **Eagle H, Oyama VI, Levy M, Horton CL, Fleischman R** 1956 The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid. *J Biol Chem* 218:607-616
321. **Inoki K, Zhu T, Guan KL** 2003 TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115:577-590
322. **Roos S, Lagerlof O, Wennergren M, Powell TL, Jansson T** 2009 Regulation of amino acid transporters by glucose and growth factors in cultured primary human trophoblast cells is mediated by mTOR signaling. *Am J Physiol Cell Physiol* 297:C723-731
323. **Eguchi S, Oshiro N, Miyamoto T, Yoshino K, Okamoto S, Ono T, Kikkawa U, Yonezawa K** 2009 AMP-activated protein kinase phosphorylates glutamine : fructose-6-phosphate amidotransferase 1 at Ser243 to modulate its enzymatic activity. *Genes Cells* 14:179-189
324. **Traxinger RR, Marshall S** 1991 Coordinated regulation of glutamine:fructose-6-phosphate amidotransferase activity by insulin, glucose, and glutamine. Role of hexosamine biosynthesis in enzyme regulation. *J Biol Chem* 266:10148-10154
325. **Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW** 2009 Select nutrients in the ovine uterine lumen. V. Nitric oxide synthase, GTP cyclohydrolase, and ornithine decarboxylase in ovine uteri and peri-implantation conceptuses. *Biol Reprod* 81:67-76
326. **Flynn NE, Meininger CJ, Haynes TE, Wu G** 2002 The metabolic basis of arginine nutrition and pharmacotherapy. *Biomed Pharmacother* 56:427-438
327. **Bird IM, Zhang L, Magness RR** 2003 Possible mechanisms underlying pregnancy-induced changes in uterine artery endothelial function. *Am J Physiol Regul Integr Comp Physiol* 284:R245-258

- 328. **Meininger CJ, Wu G** 2002 Regulation of endothelial cell proliferation by nitric oxide. *Methods Enzymol* 352:280-295
- 329. **Ishida M, Hiramatsu Y, Masuyama H, Mizutani Y, Kudo T** 2002 Inhibition of placental ornithine decarboxylase by DL-alpha-difluoro-methyl ornithine causes fetal growth restriction in rat. *Life Sci* 70:1395-1405
- 330. **Zhang X, Lin HY, Liu GY, Wang HM, Li QL, Zhu C** 2005 Expressions and regulation of endothelial and inducible nitric oxide synthases in mouse uterus during the estrous cycle and early pregnancy. *Front Biosci* 10:3172-3182
- 331. **Skarzynski G, Khamaisi M, Bursztyń M, Mekler J, Lan D, Evdokimov P, Ariel I** 2009 Intrauterine growth restriction and shallower implantation site in rats with maternal hyperinsulinemia are associated with altered NOS expression. *Placenta* 30:898-906
- 332. **Suzuki T, Ikeda Y, Yoshikawa H, Tanaka K, Morita H, Yamamoto M, Takizawa T** 2009 Gestational changes in production of NO and expression of NOS mRNA isoforms in the rat placenta. *J Vet Med Sci* 71:495-498
- 333. **Kwon H, Wu G, Meininger CJ, Bazer FW, Spencer TE** 2004 Developmental changes in nitric oxide synthesis in the ovine placenta. *Biol Reprod* 70:679-686
- 334. **Welter H, Bollwein H, Weber F, Rohr S, Einspanier R** 2004 Expression of endothelial and inducible nitric oxide synthases is modulated in the endometrium of cyclic and early pregnant mares. *Reprod Fertil Dev* 16:689-698
- 335. **Orange SJ, Painter D, Horvath J, Yu B, Trent R, Hennessy A** 2003 Placental endothelial nitric oxide synthase localization and expression in normal human pregnancy and pre-eclampsia. *Clin Exp Pharmacol Physiol* 30:376-381
- 336. **Wu G, Bazer FW, Hu J, Johnson GA, Spencer TE** 2005 Polyamine synthesis from proline in the developing porcine placenta. *Biol Reprod* 72:842-850
- 337. **Self JT, Spencer TE, Johnson GA, Hu J, Bazer FW, Wu G** 2004 Glutamine synthesis in the developing porcine placenta. *Biol Reprod* 70:1444-1451
- 338. **Hefler LA, Reyes CA, O'Brien WE, Gregg AR** 2001 Perinatal development of endothelial nitric oxide synthase-deficient mice. *Biol Reprod* 64:666-673

339. **Pallares P, Gonzalez-Bulnes A** 2008 Intrauterine growth retardation in endothelial nitric oxide synthase-deficient mice is established from early stages of pregnancy. *Biol Reprod* 78:1002-1006
340. **Tan B, Yin Y, Kong X, Li P, Li X, Gao H, Huang R, Wu G** 2009 L-Arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. *Amino Acids* 38(4):1227-1235
341. **Wu GY, Thompson JR** 1990 The effect of glutamine on protein turnover in chick skeletal muscle in vitro. *Biochem J* 265:593-598
342. **Harney JP, Bazer FW** 1989 Effect of porcine conceptus secretory proteins on interestrous interval and uterine secretion of prostaglandins. *Biol Reprod* 41:277-284
343. **Joyce MM, Burghardt JR, Burghardt RC, Hooper RN, Jaeger LA, Spencer TE, Bazer FW, Johnson GA** 2007 Pig conceptuses increase uterine interferon-regulatory factor 1 (IRF1), but restrict expression to stroma through estrogen-induced IRF2 in luminal epithelium. *Biol Reprod* 77:292-302
344. **Satterfield MC, Bazer FW, Spencer TE** 2006 Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biol Reprod* 75:289-296
345. **Spencer TE, Bazer FW** 2004 Conceptus signals for establishment and maintenance of pregnancy. *Reprod Biol Endocrinol* 2:49-64
346. **Di Renzo GC, Clerici G, Neri I, Facchinetti F, Caserta G, Alberti A** 2005 Potential effects of nutrients on placental function and fetal growth. *Nestle Nutr Workshop Ser Pediatr Program* 55:73-81; discussion 81-72
347. **Xiao XM, Li LP** 2005 L-Arginine treatment for asymmetric fetal growth restriction. *Int J Gynaecol Obstet* 88:15-18
348. **Pegg AE** 1986 Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem J* 234:249-262
349. **Sengoku K, Takuma N, Horikawa M, Tsuchiya K, Komori H, Sharifa D, Tamate K, Ishikawa M** 2001 Requirement of nitric oxide for murine oocyte maturation, embryo development, and trophoblast outgrowth in vitro. *Mol Reprod Dev* 58:262-268

350. **Reynolds LP, Borowicz PP, Caton JS, Vonnahme KA, Luther JS, Hammer CJ, Maddock Carlin KR, Grazul-Bilska AT, Redmer DA** 2009 Developmental programming: The concept, large animal models, and the key role of utero-placental vascular development. *J Anim Sci* 88:E61-72
351. **Wu G, Meininger CJ** 2009 Nitric oxide and vascular insulin resistance. *Biofactors* 35:21-27
352. **Tabor CW, Tabor H** 1984 Polyamines. *Annu Rev Biochem* 53:749-790
353. **Lange-Carter CA, Johnson GL** 1994 Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science* 265:1458-1461
354. **Bogdan C** 2001 Nitric oxide and the regulation of gene expression. *Trends Cell Biol* 11:66-75
355. **Cartwright JE, Tse WK, Whitley GS** 2002 Hepatocyte growth factor induced human trophoblast motility involves phosphatidylinositol-3-kinase, mitogen-activated protein kinase, and inducible nitric oxide synthase. *Exp Cell Res* 279:219-226
356. **Gaglioti S, Scavone C, Bevilacqua E** 2000 Participation of the mouse implanting trophoblast in nitric oxide production during pregnancy. *Biol Reprod* 62:260-268
357. **Kuzin B, Roberts I, Peunova N, Enikolopov G** 1996 Nitric oxide regulates cell proliferation during *Drosophila* development. *Cell* 87:639-649
358. **Dubey RK, Jackson EK, Luscher TF** 1995 Nitric oxide inhibits angiotensin II-induced migration of rat aortic smooth muscle cell. Role of cyclic-nucleotides and angiotensin1 receptors. *J Clin Invest* 96:141-149
359. **Gardner DK, Lane M, Spitzer A, Batt PA** 1994 Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod* 50:390-400
360. **Yao K, Yin YL, Chu W, Liu Z, Deng D, Li T, Huang R, Zhang J, Tan B, Wang W, Wu G** 2008 Dietary arginine supplementation increases mTOR signaling activity in skeletal muscle of neonatal pigs. *J Nutr* 138:867-872
361. **Kim SW, Wu G** 2009 Regulatory role for amino acids in mammary gland growth and milk synthesis. *Amino Acids* 37:89-95

362. **Luiking YC, Hallemesch MM, Lamers WH, Deutz NE** 2005 NOS3 is involved in the increased protein and arginine metabolic response in muscle during early endotoxemia in mice. *Am J Physiol Endocrinol Metab* 288:E1258-1264
363. **Sarkar S, Davies JE, Huang Z, Tunnaclyffe A, Rubinsztein DC** 2007 Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. *J Biol Chem* 282:5641-5652
364. **Rhoads JM, Corl BA, Harrell R, Niu X, Gatlin L, Phillips O, Blikslager A, Moeser A, Wu G, Odle J** 2007 Intestinal ribosomal p70(S6K) signaling is increased in piglet rotavirus enteritis. *Am J Physiol Gastrointest Liver Physiol* 292:G913-922
365. **Yang Q, Guan KL** 2007 Expanding mTOR signaling. *Cell Res* 17:666-681
366. **Dann SG, Selvaraj A, Thomas G** 2007 mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol Med* 13:252-259
367. **Bhaskar PT, Hay N** 2007 The two TORCs and Akt. *Dev Cell* 12:487-502
368. **Zhou X TM, Stone Hawthorne V et al** 2004 Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers. *Clin Cancer Res* 10:6779-6788
369. **Castellvi J, Garcia A, Rojo F, Ruiz-Marcellan C, Gil A, Baselga J, Ramon y Cajal S** 2006 Phosphorylated 4E binding protein 1: a hallmark of cell signaling that correlates with survival in ovarian cancer. *Cancer* 107:1801-1811
370. **Rojo F, Najera L, Lirola J, Jimenez J, Guzman M, Sabadell MD, Baselga J, Ramon y Cajal S** 2007 4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis. *Clin Cancer Res* 13:81-89
371. **Kimball SR, Jefferson LS** 2004 Molecular mechanisms through which amino acids mediate signaling through the mammalian target of rapamycin. *Curr Opin Clin Nutr Metab Care* 7:39-44
372. **Wu G, Pond WG, Flynn SP, Ott TL, Bazer FW** 1998 Maternal dietary protein deficiency decreases nitric oxide synthase and ornithine decarboxylase activities in placenta and endometrium of pigs during early gestation. *J Nutr* 128:2395-2402

373. **Thony B, Auerbach G, Blau N** 2000 Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J* 347 Pt 1:1-16
374. **Kimball SR, Jefferson LS** 2006 New functions for amino acids: effects on gene transcription and translation. *Am J Clin Nutr* 83:500S-507S
375. **Stipanuk MH** 2007 Leucine and protein synthesis: mTOR and beyond. *Nutr Rev* 65:122-129
376. **Drummond MJ, Rasmussen BB** 2008 Leucine-enriched nutrients and the regulation of mammalian target of rapamycin signalling and human skeletal muscle protein synthesis. *Curr Opin Clin Nutr Metab Care* 11:222-226
377. **Yang X, Yang C, Farberman A, Rideout TC, de Lange CF, France J, Fan MZ** 2008 The mammalian target of rapamycin-signaling pathway in regulating metabolism and growth. *J Anim Sci* 86:E36-50
378. **Ma XM, Blenis J** 2009 Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 10:307-318
379. **Cardenas ME, Cutler NS, Lorenz MC, Di Como CJ, Heitman J** 1999 The TOR signaling cascade regulates gene expression in response to nutrients. *Genes Dev* 13:3271-3279
380. **Hardwick JS, Kuruvilla FG, Tong JK, Shamji AF, Schreiber SL** 1999 Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc Natl Acad Sci U S A* 96:14866-14870
381. **Powers T, Walter P** 1999 Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in *Saccharomyces cerevisiae*. *Mol Biol Cell* 10:987-1000
382. **Zaragoza D, Ghavidel A, Heitman J, Schultz MC** 1998 Rapamycin induces the G0 program of transcriptional repression in yeast by interfering with the TOR signaling pathway. *Mol Cell Biol* 18:4463-4470
383. **Shi W, Meininger CJ, Haynes TE, Hatakeyama K, Wu G** 2004 Regulation of tetrahydrobiopterin synthesis and bioavailability in endothelial cells. *Cell Biochem Biophys* 41:415-434

384. **Li P, Knabe DA, Kim SW, Lynch CJ, Hutson SM, Wu G** 2009 Lactating porcine mammary tissue catabolizes branched-chain amino acids for glutamine and aspartate synthesis. *J Nutr* 139:1502-1509
385. **Palii SS, Kays CE, Deval C, Bruhat A, Fafournoux P, Kilberg MS** 2009 Specificity of amino acid regulated gene expression: analysis of genes subjected to either complete or single amino acid deprivation. *Amino Acids* 37:79-88
386. **Gardner DK, Lane M, Batt P** 1993 Uptake and metabolism of pyruvate and glucose by individual sheep preattachment embryos developed *in vivo*. *Mol Reprod Dev* 36:313-319
387. **Shah OJ, Anthony JC, Kimball SR, Jefferson LS** 2000 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. *Am J Physiol Endocrinol Metab* 279:E715-729
388. **Lee MJ, Fried SK** 2009 Integration of hormonal and nutrient signals that regulate leptin synthesis and secretion. *Am J Physiol Endocrinol Metab* 296:E1230-1238
389. **Wales RG, Waugh EE** 1993 Catabolic utilization of glucose by the sheep conceptus between days 13 and 19 of pregnancy. *Reprod Fertil Dev* 5:111-122
390. **Wales RG, Cuneo CL, Waugh EE** 1989 Incorporation of glucose by the sheep conceptus between days 13 and 19 of pregnancy. *Reprod Fertil Dev* 1:137-145
391. **Dennis PB, Jaeschke A, Saitoh M, Fowler B, Kozma SC, Thomas G** 2001 Mammalian TOR: a homeostatic ATP sensor. *Science* 294:1102-1105
392. **Morrison PJ, Hara D, Ding Z, Ivy JL** 2008 Adding protein to a carbohydrate supplement provided after endurance exercise enhances 4E-BP1 and RPS6 signaling in skeletal muscle. *J Appl Physiol* 104:1029-1036
393. **Weber FL, Jr., Fresard KM, Veach GL** 1989 Stimulation of jejunal mucosal protein synthesis by luminal glucose. Effects with luminal and vascular leucine in fed and fasted rats. *Gastroenterology* 96:935-937
394. **Jeyapalan AS, Orellana RA, Suryawan A, O'Connor PM, Nguyen HV, Escobar J, Frank JW, Davis TA** 2007 Glucose stimulates protein synthesis in skeletal muscle of neonatal pigs through an AMPK- and mTOR-independent process. *Am J Physiol Endocrinol Metab* 293:E595-603

395. **Zheng J, Wen Y, Austin JL, Chen DB** 2006 Exogenous nitric oxide stimulates cell proliferation via activation of a mitogen-activated protein kinase pathway in ovine fetoplacental artery endothelial cells. *Biol Reprod* 74:375-382

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